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(54) Title: METHOD OF DISRUPTING CELLULAR ADHESION

(57) Abstract

The present invention provides a method of inhibiting or disrupting cellular adhesion of a cell comprising transferring into the cell a polypeptide comprising a cell adhesion regulatory domain of an adhesion receptor or counter receptor expressed by the cell. In particular, the present invention provides a method of inhibiting or disrupting cellular adhesion of a cell comprising transferring into the cell a polypeptide comprising a cell adhesion regulatory domain of a subunit, i.e., the αsubunit or the βsubunit of an integrin expressed by the cell.

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METHOD OF DISRUPTING CELLULAR ADHESION

This invention was made with government support under the National Institutes of Health Grants HL45994 and HL30647. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to methods of inhibiting cellular adhesion by interfering with the functioning of surface molecules participating in the cellular adhesion. In particular, it relates to transferring into a cell to be inhibited a cellular adhesion regulatory domain peptide of an adhesion receptor or counter receptor expressed by that cell, which transfer thereby disrupts adhesion of the cell.

Background Art

Integrins are major two-way signaling receptors responsible for the attachment of cells to the extracellular matrix and for cell-cell interactions that underlie developmental programming, immune responses, tumor metastasis, and progression of atherosclerosis and thrombosis. The latter involves formation of vasoocclusive platelet thrombi bridged by fibrinogen bound to integrin $\alpha_{IIb}\beta_3$. Integrins composed of non-identical α and β subunits recognize ligands through extracellular domains and transmit intracellular signals through cytoplasmic tails. Outside-in post-ligand binding functions, such as integrin recruitment to focal adhesions and cell spreading, also depend on integrin cytoplasmic segments (1-3). Signal-dependent binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$ (Glycoprotein IIb-IIIa complex) expressed on platelets provides the key mechanism for formation of hemostatic and vasoocclusive thrombi (4). Genetic defects in integrin $\alpha_{IIb}\beta_3$ are responsible for Glanzmann's thrombasthenia, a life-long bleeding tendency arising from the inability of human platelets to bind fibrinogen. Among many mutations responsible for integrin $\alpha_{IIb}\beta_3$ dysfunction in Glanzmann's thrombasthenia, a

point mutation $Ser^{752}Pro$ in the integrin β_3 cytoplasmic tail is of particular interest (5). This "loss of function" integrin β_3 mutation exemplifies the important role of the cytoplasmic segment of integrin β_3 in regulating the adhesive function of the extracellular domain. The cytoplasmic segment of integrin β_3 comprises 41 residues from 722 through 762 (6,7). Deletion of the entire β_3 cytoplasmic segment led to the loss of adhesive function of transiently transfected Chinese Hamster Ovary (CHO) cells (8,9). A structure-function analysis of the cytoplasmic segment of integrin β_3 is needed to pinpoint its regulatory sites. Such an analysis was conducted in the present invention using a non-invasive cellular import method based on cell-permeable properties of hydrophobic (h) region of a signal peptide sequence.

Signal peptide sequences,[1a] which share the common motif of hydrophobicity, mediate translocation of most intracellular secretory proteins across mammalian endoplasmic reticulum (ER) and prokaryotic plasma membranes through the putative protein-conducting channels.[2a-11a] Alternative models for secretory protein transport also support a role for the signal sequence in targeting proteins to membranes.[12a-15a]

Several types of signal sequence-mediated inside-out membrane translocation pathways have been proposed. The major model implies that the proteins are transported across membranes through a hydrophilic protein-conducting channel formed by a number of membrane proteins.[2a-11a] In eukaryotes, newly synthesized proteins in the cytoplasm are targeted to the ER membrane by signal sequences that are recognized generally by the signal recognition particle (SRP) and its ER membrane receptors. This targeting step is followed by the actual transfer of protein across the ER membrane and out of the cell through the putative protein-conducting channel (for recent reviews, see references 2a-5a). In bacteria, the transport of most proteins across the cytoplasmic membrane also requires a similar protein-conducting channel.[7a-11a] On the other hand, signal peptides can interact strongly with lipids, supporting the proposal that the transport of some secretory proteins across cellular membranes may occur directly through the lipid bilayer in the absence of any proteinaceous channels.[14a-15a]

The present invention provides a structure-function analysis of the cytoplasmic tail of cell surface receptors based on the cellular import of synthetic peptide analogs of this region. It was determined that a peptide carrying certain residues of a subunit of the receptor could be utilized to inhibit function of the receptor. Inhibition was found to be receptor-specific. Thus the present invention provides a surprising method of inhibiting receptor function that can be very specifically directed to inhibition of a specific receptor, and thus a specific cell and a specific disease or disease-producing condition to be treated. The present invention can be utilized for a wide variety of uses, such as inhibition of adhesion and proliferation of specific cells.

SUMMARY OF THE INVENTION

The present invention provides a method of inhibiting or disrupting cellular adhesion of a cell comprising transferring into the cell a polypeptide comprising a cell adhesion regulatory domain of an adhesion receptor or counter receptor expressed by the cell. In particular, the present invention provides a method of inhibiting or disrupting cellular adhesion of a cell comprising transferring into the cell a polypeptide comprising a cell adhesion regulatory domain of a subunit, *i.e.*, the α subunit or the β subunit of an integrin expressed by the cell.

Also provided is a method of reducing or preventing an inflammatory response of a cell comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by the cell.

The present invention further provides a method of reducing or preventing blood clotting in a subject comprising contacting a platelet with a chimeric peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, thereby reducing or preventing platelet cell adhesion and reducing or preventing blood clotting in the subject.

Further provided is a method of reducing or preventing platelet adhesion in extracorporeal circulation of a subject comprising contacting *ex vivo* during the extracorporeal circulation process a platelet from the subject with a chimeric peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, and replacing the platelet into the subject, thereby reducing or preventing platelet cell adhesion in the extracorporeal circulation.

The present invention additionally provides a method of reducing or preventing platelet cell adhesion in a subject comprising contacting a platelet with a chimeric

peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, thereby reducing or preventing platelet cell adhesion in the subject.

Also provided is a method of treating or preventing coronary and/ or vascular disease or conditions in a subject comprising administering to the subject a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by a vascular endothelial cell, thereby reducing or inhibiting attachment of leukocytes and platelets to an atherosclerotic lesion.

The present invention further provides a method of reducing or preventing granulocyte adhesion in a subject comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by the granulocyte, thereby reducing or preventing adhesion of the granulocyte in the subject.

Also provided is a method of preventing or reducing restenosis in the blood vessels of a subject, comprising administering to the subject comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by an endothelial cell of the blood vessel, a granulocyte or a platelet, thereby reducing or preventing adhesion of the endothelial cells, granulocyte, and/or platelet in the subject.

Further provided by the present inventio is a method of treating adult respiratory distress syndrome in a subject comprising administering to the subject a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by a granulocyte of the subject, thereby preventing or reducing adhesion of a granulocyte to the endothelial lining of the microvasculature of the lungs and treating adult respiratory distress syndrome.

Additionally provided is a peptide comprising a cell adhesion regulatory domain of a β subunit of an integrin. The present invention further provides a peptide comprising a cell adhesion regulatory domain of an α subunit of an integrin.

Also provided by the present invention is a peptide comprising a signal peptide linked at the N-terminus to a cell adhesion regulatory domain of a β subunit of an integrin. The present invention also provides a peptide comprising a signal peptide linked at the N-terminus to a cell adhesion regulatory domain of an α subunit of an integrin.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG 1. Sequence of overlapping peptides representing the integrin β_3 cytoplasmic tail (SEQ ID NO:19), including peptides β_3-1 (SEQ ID NO:2), β_3-2 (SEQ ID NO:3) and β_3-3 (SEQ ID NO:4) and of integrin β_1 cytoplasmic tail peptide homologous to β_3-1 peptide (SEQ ID NO:6) (all in single-letter amino acid code).

FIG 2. Effect of the integrin β_3 cytoplasmic tail peptides on adhesion of HEL (A) or ECV 304 (B) cells to immobilized fibrinogen. HEL cells were preincubated in the absence (NONE) and presence of 200 μM peptide. The bars labeled with β_3-3, β_3-2, and β_3-1 represent adhesion of cells treated with non-cell-permeable peptides encompassing the integrin β_3 cytoplasmic sequence containing residues 722-737, 735-750, and 747-762, respectively. The bars labeled with β_3-3S, β_3-2S, β_3-1S, and β_3-4S represent adhesion of cells incubated with the cell-permeable peptides containing signal sequence hydrophobic region of integrin β_3 (Glycoprotein IIIa) followed by residues 722-737, 735-750, 747-762, and 742-755, respectively. Data are the mean ± standard error of the mean (SEM) from at least three independent experiments performed in triplicate. The differences in adhesion between control cells preincubated in the absence of peptides and cells treated with β_3-1S peptide are statistically significant at $p \leq 0.002$ (Student t test).

FIG 3. Intracellular location of cell-permeable β_3-1S peptide as demonstrated by confocal laser scanning microscopy (mid-cell 1μm section). Intracellular peptide was detected as yellow stains by indirect immunofluorescence assay and analyzed by a six-step Z-position sectional scanning of the cell. The top picture shows minimal staining of HEL cells treated with non-cell-permeable β_3-1 peptide. In the bottom, HEL cells treated with cell-permeable β_3-1S peptide clearly show a gain in fluorescent signal representing peptide in the cytoplasm of the HEL cells. Similar pattern was obtained with cells treated with cell-permeable β_3-2S and β_3-3S peptides. The anti-peptide β_3-1 antibody used for detection of cell-permeable β_3-1S peptide was monospecific (see Examples).

FIG 4. Inhibition of cell adhesion by cell-permeable peptides is integrin-specific and concentration-dependent. Analysis of the cell-permeable peptides β_3-1S and β_1-1S in a quantitative adhesion assay of HEL cells (A), ECV 304 cells (B) and HF cells (C). HEL and ECV 304 cells adhered to immobilized fibrinogen and HF cells adhered to uncoated plastic. Import of peptides and cell adhesion assay were performed as in FIG 2. Data are the mean ± SEM from at least three experiments performed in triplicate. The differences between β_3-1S peptide and β_1-1S peptide at 200μM were significant at $p < 0.0001$ for both HEL and ECV 304 cells. The difference between β_3-1S peptide and β_1-1S peptide at 200μM were significant at $p<0.0001$ for the HF cell line.

FIG 5. Effect of cell-permeable mutant peptides β_3-1S ($Ser^{752}Pro$) and β_3-1S ($Ser^{752}Ala$) on adhesion of HEL cells (○,□,△) and ECV 304 cells (●,■,▲) to immobilized fibrinogen. Import of peptides and the cell adhesion assay were performed as in FIG 2. Data are the mean ±SEM from at least three experiments performed in triplicate. The differences between β_3-1S peptide and its mutant β_3-1S ($Ser^{752}Pro$) were significant at $p < 0.0001$ for both HEL and ECV 304 cells. The difference between β_3-1S and β_3-1S ($Ser^{752}Ala$) was not significant.

FIG 6. Effect of cell-permeable mutant peptides β_3-1S ($Tyr^{747}Phe$), β_3-1S ($Tyr^{759}Phe$), and β_3-1S ($Tyr^{747/759}Phe$) on adhesion of HEL (A) and ECV 304 (B) cells to immobilized fibrinogen. Import of peptides and the cell adhesion assay were performed as in FIG 2. Data are the mean ±SEM from at least three experiments performed in triplicate. The differences between β_3-1S peptide and all three β_3-1S mutant Tyr→Phe peptides were significant at $p < 0.0001$ for both HEL and ECV 304 cells.

FIG. 7 shows a schematic presentation of the approach to the structure-function analysis of cytoplasmic tails, here specifically of integrin α_{IIb} β_3 subunits. Sequences of hydrophobic signal peptide (SEQ ID NO:1), and peptides β_3-1 (SEQ ID NO:2), β_3-2 (SEQ ID NO:3), β_3-3 (SEQ ID NO:4) and β_3-4 (SEQ ID NO:5) are shown.

FIG. 8 shows the effect of cell-permeable peptide β_3-1S in the absence (•) or the presence (▪) of verapamil in HEL cells.

FIG. 9 shows inhibition of HEL cell adhesion to fibrinogen by cell-permeable peptides β_3-1S and α_{IIb} is concentration dependent.

FIG. 10 shows the β_2 peptide and β_2-3 (residues 724-741; SEQ ID NO:9), β_2-2 (residues 7740-756; SEQ ID NO:8) and β_2-1 (SEQ ID NO:7) peptides, as well as signal sequence (SEQ ID NO:1). Additional β_2 peptides are β_2-4 (LFKSATTTVMNPKFAES (residues 753-769; SEQ ID NO:20)) and β_2-5 (KEKLKSQWNNDNPLF (residues 740-754; SEQ ID NO:21)).

DETAILED DESCRIPTION OF THE INVENTION

As used in the specification and in the claims, "a" can mean one or more, depending upon the context in which it is used.

Analysis of the 41 residue cytoplasmic tail of integrin β_3 was undertaken by applying our recently developed cell-permeable peptide import technique (10) to probe integrin β_3 cytoplasmic protein-protein interactions. As a functional endpoint, adhesion of human erythroleukemia (HEL) cells to immobilized fibrinogen in response to stimulation with 4β-phorbol 12-myristate 13-acetate (PMA) was used. HEL cells express endogenous integrin $\alpha_{IIb}\beta_3$ and serve as a useful model for structure-function studies of platelet constituents (11,12). The integrin β_3 is also expressed as a heterodimer with integrin α_v in human platelets and endothelial cells (13). Therefore, adhesion of the ECV 304 cell line derived from human umbilical vein endothelial cells that express $\alpha_v\beta_3$ integrin (vitronectin receptor) (14) was studied. Using cell-permeable peptides representing wild-type and mutated sequences, the present invention identifies the major Cell Adhesion Regulatory Domain (CARD) of integrin β_3 . It encompasses a 16 amino acid sequence of its cytoplasmic tail. A synthetic peptide mimetic representing CARD imported by HEL and ECV 304 cells inhibits "from within" their adhesion to immobilized fibrinogen by competing with intracellular protein-protein interactions involving the integrin β_3 cytoplasmic tail.

The present invention reports the structure-function analysis of the cytoplasmic tail of integrin subunits β_1, β_2 , β_3 and α_{IIb} based on the cellular import of synthetic peptide analogs of this region. Among the four overlapping cell-permeable peptides of β_3 only the peptide carrying residues 747-762 of the carboxy-terminal segment of integrin β_3 inhibited adhesion of human erythroleukemia (HEL) cells and of human endothelial cells (ECV) 304 to immobilized fibrinogen mediated by integrin β_3 heterodimers, $\alpha_{IIb}\beta_3$, and $\alpha_v\beta_3$, respectively. Inhibition of adhesion was integrin-specific because the cell-permeable β_3 peptide (residues 747-762) did not inhibit adhesion of human fibroblasts mediated by integrin β_1 heterodimers. Conversely, a cell-permeable

peptide representing homologous portion of the integrin β_1 cytoplasmic tail (residues 788-803) inhibited adhesion of human fibroblasts, whereas it was without effect on adhesion of HEL or ECV 304 cells.

The cell-permeable integrin β_3 (Glycoprotein IIIa) peptide (residues 747-762) carrying a known loss-of-function mutation ($Ser^{752}Pro$) responsible for the genetic disorder Glanzmann's thrombasthenia Paris I did not inhibit cell adhesion of HEL or ECV 304 cells, while the β_3 peptide carrying a $Ser^{752}Ala$ mutation was inhibitory. Although Ser^{752} is not essential, Tyr^{747} and Tyr^{759} form a functionally active tandem because conservative mutations $Tyr^{747}Phe$ or $Tyr^{759}Phe$ resulted in a non-functional cell permeable integrin β_3 peptide. It is herein demonstrated that the carboxy terminal segment of the integrin β_3 cytoplasmic tail spanning residues 747-762 constitutes a major intracellular Cell Adhesion Regulatory Domain (CARD) that modulates the adhesion of integrin β_3-expressing cells with immobilized fibrinogen. It is further demonstrated that the carboxy terminal segment of the integrin β_1 cytoplasmic tail (residues 788-803) constitutes a major intracellular Cell Adhesion Regulatory Domain (CARD) that modulates the adhesion of integrin β_1-expressing cells. Additionally shown is a CARD of the integrin β_2 subunit is within a peptide comprising amino acids 724-769. Furthermore, a cytoplasmic segment containing amino acids 989-1008 of integrin subunit α_{IIb}. was tested. Import of cell-permeable peptides carrying a CARD domain results in inhibition "from within" of the adhesive function of these integrins in cells expressing these integrins. The present invention includes the basic premise that the cytoplasmic segment of the integrin β_2 subunit and its α subunit counterparts, α_L, α_M, and α_X, carry a functionally related CARD, and that the cytoplasmic segment of the integrin β_1 subunit and its α subunit counterparts, carry a functionally related CARD. A CARD for any selected adhesion receptor, adhesion molecule or counter receptor can readily be developed following the procedures herein.

The present invention provides a method of inhibiting adhesion of a cell comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by the cell.

The adhesion receptor can be an integrin. When the adhesion receptor is an integrin, the subunit can be a β subunit of an integrin. In particular, the β subunit can be β_3, β_2, or β_1, or example. The subunit can be an α subunit of an integrin. In particular it can be, for example, α_{IIb} subunit.

The adhesion receptor can be a selectin. The adhesion receptor can be a cell adhesion molecule such as an ICAM, *e.g.*, ICAM-1. ICAM-2, ICAM-3.

The present invention provides a method of inhibiting or disrupting cellular adhesion of a cell comprising transferring into the cell a polypeptide comprising a cell adhesion regulatory domain of an adhesion receptor or counter receptor expressed by the cell. In particular, the present invention provides a method of inhibiting or disrupting cellular adhesion of a cell comprising transferring into the cell a polypeptide comprising a cell adhesion regulatory domain of a subunit, *i.e.*, the α subunit or the β subunit of an integrin expressed by the cell.

In particular, the present invention provides a method of reducing or preventing excessive proliferation of a fibroblast comprising transferring into the fibroblast a peptide comprising a cell adhesion regulatory domain of a β_1 subunit of an integrin receptor. The administration of the peptide disrupts adhesion, thus reducing or preventing adhesion-dependent proliferation of the cells. For example, a peptide comprising the amino acid sequence set forth in SEQ ID NO:6 (peptide β_1-1) can be used in this method. Such inhibition of excessive fibroblast proliferation can be used to promote wound healing.

Because adhesion receptors and counter receptor mediate various forms of cellular adhesion, the present method can be utilized to disrupt these various forms of adhesion. Therefore, for example, the present method can be used to disrupt cell-substratum adhesion, cell-cell aggregation, and/or direct cell-cell adhesion. The present method can be used to transfer the peptide into a cell *in vitro*; *ex vivo*, to ultimately transfer the cell into a subject; and *in vivo* directly into a cell in a subject.

Several integrins and the cells expressing particular integrins are known in the art (*see*,, *e.g.*, Hynes, R.O. 1992 *Cell 69*:11-25; Felding-Habermann, B. and D.A. Cheresh 1993 *Curr. Opin. Cell Biol. 5*:863-868; Albeda and Buck 1990 *FASEB J. 4*:2868-2880; Arnaout 1990 *Blood 75*:1037-1050; Hemler 1990 *Annu. Rev. Immunol.8*:365-400; Springer 1990 *Nature 346*:425-434; Ruoslahti 1991 *J. Clin Invest. 87*:1-5). An integrin is an αβ heterodimer. The α subunits vary in size (between about 120 and 180 kD) and are each covalently associated with a β subunit. Currently about 8 β subunits are characterized and about 14 α subunits are characterized. Thus, integrins can include an α chain selected from, for example, vertebrate α_V, α_1, α_2, α_3, α_4,α_5, α_6, α_7, α_8, α_L, α_M, α_{IIb}, α_{IEL}, and α_X, and a β chain from, for example, β_1, β_2, β_3, β_4, β_6, β_7, and β_8. Thus, for example, a cell adhesion regulatory domain of a β_1 subunit can be used to disrupt cellular adhesion of a cell expressing, for example, $\alpha_1\beta_1$ to $\alpha_8\beta_1$ or $\alpha_V\beta_1$ integrin. Thus, for example, a cell adhesion regulatory domain of a β_2 subunit can be used to disrupt cellular adhesion of a cell expressing, for example, $\alpha_L\beta_2$, $\alpha_M\beta_2$, or $\alpha_X\beta_2$ integrin. Also, for example, a cell adhesion regulatory domain of a β_3 subunit can be used to disrupt cellular adhesion of a cell expressing, for example, $\alpha_{IIb}\beta_3$ or $\alpha_V\beta_3$ integrin. Additionally, for example, a cell adhesion regulatory domain of a β_1 subunit can be used to disrupt cellular adhesion of a cell expressing, for example, $\alpha_5\beta_1$ or $\alpha_2\beta_1$. Furthermore, a cell adhesion regulatory domain of an α_{IIb} subunit can be used to inhibit, for example, $\alpha_{IIb}\beta_3$ integrin. The present method can disrupt cellular adhesion of an integrin regardless of the extracellular binding region of the receptor and regardless of the recognition sequence on the target protein recognized by the integrin receptor because the present method disrupts binding by disrupting the intracellular domain of the receptor. Therefore, the binding sites for the receptors need not be fully characterized.

Most integrins are expressed on a wide variety of cells. Most cells express several integrins. The present invention can be utilized for any cell type as long as the polypeptide comprising the appropriate cell adhesion regulatory domain can be transferred into the cell and the cell expresses the integrin. Mechanisms particularly effective for transferring such proteins (by means of transferring the protein or the nucleic acid encoding it) are known in the art for specific cell types, and additionally

some are exemplified herein. (*see, e.g.*, Hynes, R.O. 1992 Cell 69:11-25; Felding-Habermann, B. and D.A. Cheresh 1993 *Curr. Opin. Cell Biol. 5*:863-868; Albeda and Buck 1990 *FASEB J. 4*:2868-2880)

Selectins, cell surface adhesion molecules, for example, L-selectin, (Tedder TF, et al.J. Experimental Medicine 1995 June;181:2259-2264), can also be inhibited by this method to disrupt cellular adhesion. The CARD of any given selectin can be readily determined following the methods taught herein as exemplified by determining the CARDs of the β_1 and β_3 subunits of integrins. Briefly, one can introduce cytoplasmic segments into appropriate cells expressing the receptor or counter receptor and test for inhibition of adhesion as demonstrated in the examples. Using selectin CARD peptides, one can target adhesion disruption specifically in cells expressing the particular selectin. For example, L-selectin is expressed by most leukocytes, E-selectins are expressed by endothelial cells, and P-selectins are expressed by platelets.

Cadherins, calcium-dependent adhesion receptors, can also be inhibited by this method to disrupt cellular adhesion. The CARD of any given cadherin, such as E-cadherin, N-cadherin and P-cadherin, can be readily determined following the methods taught herein as exemplified by determining the CARDs of the β_1 and β_3 subunits of integrins. Briefly, one can introduce cytoplasmic segments into appropriate cells expressing the receptor or counter receptor and test for inhibition of adhesion as demonstrated in the examples. Using cadherin CARD peptides, one can target adhesion disruption specifically in cells expressing the particular selectin. For example, E-cadherins are expressed by endothelial cells, N-cadherins are expressed on neuronal cells and P-cadherins are expressed by platelets.

Cell adhesion molecules such as ICAMs can also be used in the present method. ICAM CARDs can readily be determined following the methods presented herein. Using ICAM CARD peptides, one can target adhesion disruption specifically in cells expressing the particular ICAM, for example, epithelial cells, endothelial cells, or leukocytes.

Transfer of the polypeptide into the cell can be accomplished by any selected means. For example, a signal peptide can be linked to a polypeptide comprising the cell adhesion regulatory domain and this complex administered to the cell. The signal peptide can be linked, e.g., by constructing a recombinant vector encoding a chimeric peptide comprising the signal peptide and the polypeptide comprising the cell adhesion regulatory domain. This recombinant vector can then be expressed *ex vivo*, after which the protein would be transferred into the cell to be disrupted, aided in cell entry by the signal peptide. This mode of transfer is exemplified in the examples herein. The signal peptide can also be linked to the polypeptide by other means such as a chemical link (see, e.g., U.S. Serial No. 08/258,852).

Furthermore, other hydrophobic sequences other than signal peptides, can be used to deliver the peptide into the cell. Helical structures in homeobox proteins can be used. Any such peptide for transfer of the peptide into a cell can be generated as part of a chimeric peptide; it can be linked by a peptide (amide) bond; it can be cross-linked chemically.

Additionally, a nucleic acid encoding the peptide comprising the cell adhesion regulatory domain can be constructed such that the coding region is functionally linked to a promoter compatible with the cell in which adhesion is to be disrupted, and the vector then transferred into the cell. The cell can then produce the peptide itself. If such *in vivo* expression is utilized, the vector can be selected such that the vector can be readily transferred into the specific cell type. For example, an adenoviral vector, an adeno-associated viral vector or a retroviral could be utilized to transfect any of several types of cells. Other viral vectors have cell specificity as known in the art, and can be utilized accordingly for targeting such cells.

A suitable nucleotide sequence for a nucleic acid encoding a CARD peptide can readily be deduced from the amino acid sequence of the CARD, as is standard in the art. Additionally, species-preferred codons can be utilized for the particular species whose

cell is to be transferred for optimal expression of the peptide. Such species codon specificities are known in the art.

Alternatively other transfection means can be utilized separately or in conjunction with that exemplified herein to achieve global or selective transfer of the vector. For example, a cationic liposome composition comprising the CARD peptide alone, the chimeric signal peptide-CARD peptide, or a nucleic acid encoding the CARD peptide can be utilized particularly to target the lungs, upon inhalation or intravenous injection (Brigham, *et al. Amer. J. Respir. Cell and Mol. Biol.* 8:209-213 (1993); Felgner *et al.*, 1987 *Proc. Natl. Acad. Sci. U.S.A.* **84**:7413; U.S. Patent No. 4,897,355 (Eppstein, et al.)) . By way of another example, liposome compositions can be utilized that have incorporated proteins that have specific receptors on the target cells. Additionally, the promoter of the vector can also be selected such that expression from it can be induced or expressed in only particular cell types. Inducible promoters include, for example, the metallothionein promoter, which can be induced by exposure to zinc, such as in the diet of the subject. Many such selectively or inducibly expressed promoters are known in the art.

Cationic and anionic liposomes are contemplated for use in this invention, as well as liposomes having neutral lipids. Cationic liposomes can be complexed with the CARD peptide, the signal peptide-CARD peptide chimeric peptide, or the nucleic acid encoding the CARD peptide by mixing these components and allowing them to charge-associate. Cationic liposomes are particularly useful with a nucleic acid because of the nucleic acid's negative charge. Examples of cationic liposomes include lipofectin, lipofectamine, lipofectace and DOTAP.[32a-34a] Anionic liposomes generally are utilized to encase within the liposome the substances to be delivered to the cell. Procedures for forming cationic liposomes encasing substances are standard in the art[35a] and can readily be utilized herein by one of ordinary skill in the art to encase the complex of this invention.

For peptide import using an importation-competent signal peptide, suitable import conditions are exemplified herein and include cell and complex temperature between about 18°C and about 42°C, with a preferred temperature being between about 22°C and about 37°C. For administration to a cell in a subject, the complex, once in the subject, will of course adjust to the subject's body temperature. For *ex vivo* administration, the complex can be administered by any standard methods that would maintain viability of the cells, such as by adding it to culture medium (appropriate for the target cells) and adding this medium directly to the cells. As is known in the art, any medium used in this method can be aqueous and non-toxic so as not to render the cells non-viable. In addition, it can contain standard nutrients for maintaining viability of cells, if desired. For *in vivo* administration, the complex can be added to, for example, a blood sample or a tissue sample from the patient, or to a pharmaceutically acceptable carrier, e.g., saline and buffered saline, and administered by any of several means known in the art. Examples of administration include parenteral administration, e.g., by intravenous injection including regional perfusion through a blood vessel supplying the tissues(s) or organ(s) having the target cell(s), or by inhalation of an aerosol, subcutaneous or intramuscular injection, topical administration such as to skin wounds and lesions, direct transfection into, e.g., bone marrow cells prepared for transplantation and subsequent transplantation into the subject, and direct transfection into an organ that is subsequently transplanted into the subject. Further administration methods include oral administration, particularly when the composition is encapsulated, or rectal administration, particularly when the composition is in suppository form. A pharmaceutically acceptable carrier includes any material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the selected complex without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

Administration can be performed for a time length of about 1 minute to about 72 hours. Preferable time lengths are about 5 minutes to about 48 hours, and even more preferably about 5 minutes to about 20 hours, and even more preferably

about 5 minutes to about 2 hours. Optimal time lengths and conditions for any specific complex and any specific target cell can readily be determined, given the teachings herein and knowledge in the art.[27a] Specifically, if a particular cell type *in vivo* is to be targeted, for example, by regional perfusion of an organ or tumor, cells from the target tissue can be biopsied and optimal dosages for import of the complex into that tissue can be determined *in vitro*, as described herein and as known in the art, to optimize the *in vivo* dosage, including concentration and time length. Alternatively, culture cells of the same cell type can also be used to optimize the dosage for the target cells *in vivo*.

For either *ex vivo* or *in vivo* use, the complex can be administered at any effective concentration. An effective concentration is that amount that results in importation of the biologically active molecule into the cell. Such a concentration will typically be between about 0.5 nM to about 100 μM (culture medium concentration (*ex vivo*) or blood serum concentration (*in vivo*)). Optimal concentrations for a particular complex and/or a particular target cell can be readily determined following the teachings herein. Thus, *in vivo* dosages of the complex include those which will cause the blood serum concentration of the complex to be about 0.5 nM to about 100 μM. A preferable concentration is about 2 nM to about 50 μM. The amount of the complex administered will, of course, depend upon the subject being treated, the subject's age and weight, the manner of administration, and the judgment of the skilled administrator. The exact amount of the complex will further depend upon the general condition of the subject, the severity of the disease/condition being treated by the administration and the particular complex chosen. However, an appropriate amount can be determined by one of ordinary skill in the art using routine optimization given the teachings herein.

Parenteral administration, e.g., regional perfusion, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, such as liquid solutions, suspensions, or emulsions. A slow release or sustained release system, such as disclosed in U.S. Patent No. 3,710,795, can also be used, allowing the maintenance of a constant level of dosage.

Depending on the intended mode of administration, the pharmaceutical compositions may be in the form of solid, semi-solid or liquid dosage forms, such as, for example, tablets, suppositories, pills, capsules, powders, liquids, suspensions, lotions, creams, gels, or the like, preferably in unit dosage form suitable for single administration of a precise dosage. The compositions will include, as noted above, an effective amount of the selected drug in combination with a pharmaceutically acceptable carrier and, in addition, may include other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc.

For solid compositions, conventional nontoxic solid carriers include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talc, cellulose, glucose, sucrose, magnesium carbonate, and the like. Liquid pharmaceutically administrable compositions can, for example, be prepared by dissolving, dispersing, etc. an active compound as described herein and optional pharmaceutical adjuvants in an excipient, such as, for example, water, saline, aqueous dextrose, glycerol, ethanol, and the like, to thereby form a solution or suspension. If desired, the pharmaceutical composition to be administered may also contain minor amounts of nontoxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see *Remington's Pharmaceutical Sciences*.[27a]

An "importation competent signal peptide," as used herein, is a sequence of amino acids generally of a length of about 10 to about 50 or more amino acid residues, many (typically about 55-60%) residues of which are hydrophobic such that they have a hydrophobic, lipid-soluble portion.[1a] The hydrophobic portion is a common, major motif of the signal peptide, and it is often a central part of the signal peptide of protein secreted from cells. A signal peptide is a peptide capable of penetrating through the cell membrane to allow the export of cellular proteins. The signal peptides of this invention, as discovered herein, are also "importation competent," i.e., capable of penetrating through the cell membrane from outside the cell to the interior of the cell. The amino

acid residues can be mutated and/or modified (i.e., to form mimetics) so long as the modifications do not affect the translocation-mediating function of the peptide. Thus the word "peptide" includes mimetics and the word "amino acid" includes modified amino acids, as used herein, unusual amino acids, and D-form amino acids. All importation competent signal peptides encompassed by this invention have the function of mediating translocation across a cell membrane from outside the cell to the interior of the cell. Such importation competent signal peptides could potentially be modified such that they lose the ability to export a protein but maintain the ability to import molecules into the cell. A putative signal peptide can easily be tested for this importation activity following the teachings provided herein, including testing for specificity for any selected cell type.

Signal peptides can be selected, for example, from the SIGPEP database, which also lists the origin of the signal peptide.[30a, 38a] When a specific cell type is to be targeted, a signal peptide used by that cell type can be chosen. For example, signal peptides encoded by a particular oncogene can be selected for use in targeting cells in which the oncogene is expressed. Additionally, signal peptides endogenous to the cell type can be chosen for importing biologically active molecules into that cell type. And again, any selected signal peptide can be routinely tested for the ability to translocate across the cell membrane of any given cell type according to the teachings herein. Specifically, the signal peptide of choice can be conjugated to a peptide, *e.g.*, a CARD of a cellular adhesion receptor, adhesion molecule or counter receptor, and administered to a cell, and the cell is subsequently screened for the presence of the CARD peptide.

The presence of modified amino acids in the signal peptide can additionally be useful for rendering a peptide, polypeptide or protein more resistant to peptidases in the subject. Thus these signal peptides can allow for more effective treatment by allowing more peptides to reach their target and by prolonging the life of the peptide before it is degraded. Additionally, one can modify the amino acid sequence of the signal peptide to alter any proteolytic cleavage site present in the original signal sequence for removing the signal sequence. Cleavage sites are characterized by small,

positively charged amino acids with no side chains and are localized within about 1 to about 4 amino acids from the carboxy end of the signal peptide.[1a]

An example of a useful signal peptide is the signal peptide from human integrin β_3: VTVLALGALAGVGVG (SEQ ID NO:1) (hydrophobic region (h-region) of the signal peptide sequence of human integrin subunit β_3) (6,7). Another example is the signal peptide from Kaposi fibroblast growth factor (K-FGF)[16a-17a] : Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro (SEQ ID NO:17). Any signal peptide, however, capable of translocating across the cell membrane into the interior of the selected target cell can be used according to this invention.

The cell into which the CARD peptide is transferred can be any cell expressing the adhesion molecule or counter receptor. For example, the cell can be a white blood cell; a granulocyte, a monocyte; a lymphocyte; it can also be a cancer cell, specifically a tumor cell, a leukemia cell. Cancer cell includes cells of a tumor mass and metastatic cells. Also for β_3: platelets, endothelial cells; for β_1: fibroblasts. Cells can be within a tissue or organ, for example, supplied by a blood vessel into which the complex is administered. Additionally, the cell can be targeted by, for example, inhalation of the molecule linked to the peptide to target the lung epithelium, or ingestion or suppository administration to target the intestinal epithelium. In addition, the chimeric peptide or nucleic acid encoding the CARD peptide can be administered directly to a tissue site in the body. As discussed above, the signal peptide utilized can be chosen from signal peptides known to be utilized by the selected target cell, or a desired signal peptide can be tested for importing ability given the teachings herein. Generally, however, all signal peptides have the common ability to cross cell membranes due, at least in part, to their hydrophobic character. Thus, in general, a membrane-permeable signal peptide can be designed and used for any cell type, since all eukaryotic cell membranes have a similar lipid bilayer.

The effective concentration of imported peptide or its mimetic is an amount sufficient to inhibit cellular adhesion. Experimentally, one can calculate the

concentration of CARD peptide necessary *in vitro* to cause 50% inhibition of, *e.g.*, fibroblast or endothelial cells.

Additionally, the concentration needed to achieve sufficient transfer can be reduced by employing methods to increase intracellular concentration of peptides. For example, as shown herein, cells can be treated with a compound that prevents active transport of peptides from the cell. Herein it is shown that blockers of the MDR pump, such as chemotherapeutic drug analogs, calcium channel antagonists (*e.g.*, verapamil), immunosuppressive cyclic peptides (*e.g.*, cyclosporin), and calmodulin inhibitors also block the removal of peptides from the cell. Thus, in combination with the treatment of cells with blockers of MDR pump or similar membrane proteins that remove drugs, peptides, etc. from cells, the amount of CARD peptide to be administered can be reduced. Thus, effectiveness of imported peptides can be increased while any potential side effects can be minimized. Often a subject may already be taking an MDR pump blocker; thus this combination treatment can have minimal adverse effect. The amount of blocker can be readily determined using standard methods, in particular as exemplified herein. A typical dosage will be that amount used in the art for verapamil or cyclosporin administration.

The cell adhesion regulatory domain of an adhesion receptor or adhesion molecule, such as an integrin, a cadherin or a selectin, or of a counter receptor is a peptide, or mimetic thereof, comprising the cytoplasmic portion of a subunit of the adhesion receptor, adhesion molecule or counter receptor which, when transferred into a cell expressing the adhesion receptor, adhesion molecule or counter receptor from which the peptide was derived, inhibits binding of the adhesion receptor or adhesion molecule to its ligand or counter receptor or of the counter receptor to its corresponding receptor. This portion is herein referred to as the cytoplasmic adhesion regulatory domain (CARD). For example, for an integrin, which has an α subunit and a β subunit, a composition comprising a peptide comprising a CARD of the α subunit can be used or a composition comprising a peptide comprising a CARD of the β subunit can be used. For example, for a receptor having the integrin β_3 chain, a peptide comprising amino

acids 747-762 of the integrin β_3 chain can be used: YKEATSTFTNITYRGT (SEQ ID NO:2- residues 747-762 of integrin subunit β_3 (peptide β_3-1)) or VTVLALGALAGVGVGYKEATSTFTNITYRGT (SEQ ID NO:11-signal sequence linked to residues 747-762 of integrin subunit β_3 at N-terminus (peptide β_3-1S)); for a receptor having the integrin β_1 chain, a peptide comprising amino acids 788-803 of the integrin β_1 chain can be used: YKSAVTTVVNPKYEGK (SEQ ID NO:6- residues 788-803 of integrin subunit β_1 (peptide β_1-1)) or VTVLALGALAGVGVGYKSAVTTVVNPKYEGK (SEQ ID NO:12- signal sequence linked to residues 788-803 of integrin subunit β_1 at N-terminus (peptide β_1-1S)); for a receptor having the integrin β_2 chain, a peptide comprising amino acids 724-769 of the integrin β_2 chain can be used: KALIHLSDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES (SEQ ID NO:18- residues 724-769 of integrin subunit β_2). A shorter β_2 peptide retaining cell adhesion inhibiting activity can be readily derived following the examples herein. For example, peptides β_2-1 (SEQ ID NO:7), β_2-2 (SEQ ID NO:8), β_2-3 (SEQ ID NO:9), β_2-4 (SEQ ID NO:20), or β_2-5 (SEQ ID NO:21) can be used in the provided methods to further delineate the integrin β_2 CARD. For a receptor having the integrin α_{IIb} subunit, a peptide comprising amino acids 989-1008 can be used: KVGFFKRNRPPLEEDDEEGE (SEQ ID NO:10- peptide α_{IIb} (residues 989-1008 of integrin subunit α_{IIb})) or VTVLALGALAGVGVGKVGFFKRNRPPLEEDDEEGE (SEQ ID NO:14- signal sequence linked to residues 989-1008 of integrin subunit α_{IIb} at N-terminus (peptide α_{IIb} S)). For integrins α_L, α_M, and α_X and ICAM-1, ICAM-2, ICAM-3, and L-selectin, E-selectin, and P-selectin, or any other adhesion receptor or counter receptor, cytoplasmic segments of the subunit can be used as adhesion inhibitory peptides when imported into cells expressing the receptor or counter receptor. Additionally, shorter peptides can be tested for such activity and used.

A peptide comprising the cell adhesion regulatory domain can have preferably conservative amino acid substitutions and or deletions as long as the domain retains its ability to inhibit binding of the receptor to its ligand and/or counter receptor. Useful substitutions and deletions can be readily determined by following the teaching of the

examples. For example, as found by the methods in the Examples, a polypeptide including amino acids 747-762 of the integrin β_3 chain can include a Serine to Alanine substitution at amino acid 752; however, a Serine to Proline substitution at amino acid 752, a less conservative substitution, results in a peptide that is inactive in the function of inhibiting binding of the receptor to its ligand. By following this method, for which an example of an acceptable substitution and an unacceptable substitution is provided, other acceptable substitutions can be obtained and thus such substituted peptides are within the scope of this invention. Additionally, as known in the art, conservative and non-conservative substitutions can be predicted.

A mimetic of a CARD peptide can be designed based upon the conformational characteristics of the CARD peptide. For example, the CARD peptide can be crystallized and the characteristics of the peptide determined using a computer program designed for such functions. A computer program can then be run to predict substitutions in the molecule that can be made for specific amino acids in the peptide to retain the binding conformation. The resulting mimetic can be a peptide mimetic or it can have other molecules substituted in place of at least one or more of the amino acids. Mimetics can be designed, for example, to be more resistant to degradation in the body.

The present invention further provides a method of reducing or preventing an inflammatory response of a cell comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor, adhesion molecule or counter receptor expressed by the cell. The adhesion receptor, adhesion molecule or counter receptor can be any adhesion receptor, adhesion molecule or counter receptor expressed by the target cell of interest. For example, the adhesion receptor can be an integrin. The subunit can be a β subunit of an integrin expressed by the cell. The subunit can be an α subunit of an integrin expressed by the cell.

The present invention further provides a method of reducing or preventing blood clotting in a subject comprising contacting a platelet with a chimeric peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory

domain (CARD) of a β subunit of an integrin, thereby reducing or preventing platelet cell adhesion and reducing or preventing blood clotting in the subject. In particular, the CARD can be of a β1, β2, or a β3 subunit of an integrin.

The present invention further provides a method of reducing or preventing blood clotting in a subject comprising contacting *ex vivo* a platelet from the subject with a chimeric peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, and replacing the platelet into the subject, thereby reducing or preventing platelet cell adhesion and reducing or preventing blood clotting in the subject.

The instant invention additionally provides a method of reducing or preventing platelet cell adhesion in a subject comprising contacting a platelet with a chimeric peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, thereby reducing or preventing platelet cell adhesion in the subject.

The peptide can be administered *in vivo* or *ex vivo* to cells removed from the subject and then injected back into the subject once the peptide is transferred. For example, if a patient is undergoing a procedure such as pheresis or dialysis, the peptide can be administered to the blood as it is removed. If a subject is undergoing a procedure such as a removal of a tumor, the peptide can be directly infused to the site of tumor removal, to inhibit regrowth of the tumor and to inhibit metastasis. Furthermore, if a subject is undergoing open heart surgery or angioplasty, the peptide can be administered directly at the site of injury to the heart and/or blood vessel, to prevent inflammation or restenosis. A subject with adult respiratory distress syndrome can be administered by inhalation of the peptide in a liposome composition in a manner and in dosages similar to that used for applying CFTR to the lungs in cystic fibrosis patients. Also, for adult respiratory distress syndrome, the peptide can be administered intravenously. Furthermore, for intestinal applications, the peptide can be placed in a carrier resistant to

pepsin and taken orally; alternatively the peptide can be administered such that it proceeds directly to the intestine with minimal dwell time in the stomach.

If a patient is undergoing a procedure involving extracorporeal circulation of the patient's blood, the present method can be used to prevent platelet adhesion in the subject's blood while it is circulating in the equipment for the extracorporeal circulation. Therefore, the present invention provides a method of reducing or preventing platelet adhesion in extracorporeal circulation of a subject comprising contacting *ex vivo* during the extracorporeal circulation process a platelet from the subject with a chimeric peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, and replacing the platelet into the subject, thereby reducing or preventing platelet cell adhesion in the extracorporeal circulation.

The present invention provides a method of treating or preventing coronary and/or vascular disease or conditions in a subject comprising administering to the subject a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by vascular endothelial cells, thereby reducing or inhibiting attachment of leukocytes and platelets to an atherosclerotic lesion.

It is preferred that the peptide be administered prior to formation of the lesion. For example, it can be administered at the time of surgery, such as angioplasty or open heart surgery, is performed on a blood vessel or on the heart.

The present invention provides a method of reducing or preventing granulocyte adhesion in a subject comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by the granulocyte, thereby reducing or preventing adhesion of the granulocyte in the subject.

This method can be performed *in vivo* or *ex vivo*. One can, for example, to transfer the peptide into the cell, contact a granulocyte of the subject with a chimeric peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, thereby reducing or preventing granulocyte adhesion in the subject.

Administration is preferably performed at the time of a surgical procedure associated with granulocyte adhesion occurring after the procedure, such as open heart surgery or angioplasty. Granulocytes and platelets adhere to damaged endothelial cells lining the blood vessels, especially in the microvasculature of the lungs; therefore, this treatment can be used to selectively affect areas having damaged endothelial cells.

The present invention provides a method of preventing or reducing atherosclerotic plaques in the blood vessels of a subject, comprising administering to the subject comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor, adhesion molecule or counter receptor expressed by an endothelial cell of the blood vessel, a granulocyte or a platelet, thereby reducing or preventing adhesion of the endothelial cells, granulocyte, and/or platelet in the subject. Such a method can be performed *in vivo* or *ex vivo* to cells removed from the subject.

The present invention provides a method of treating adult respiratory distress syndrome in a subject comprising administering to the subject a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by a granulocyte of the subject, thereby preventing or reducing adhesion of a granulocyte to the endothelial lining of the microvasculature of the lungs and treating adult respiratory distress syndrome.

Granulocyte binding to endothelial cells in blood vessels of the lungs inhibits gas exchange in the capillaries to cause adult respiratory distress syndrome and can further complicate with peptic shock. Thus, by preventing or reducing the binding of

granulocytes to the blood vessels, the respiratory distress response can be inhibited or reduced.

In any of the methods wherein a CARD peptide is transferred into a cell, the peptide can be transferred by any of several means. For example, it can be prepared in a chimeric peptide which includes at its amino-terminal end an importation-competent signal peptide. It can also be linked to an importation competent signal peptide by other means and administered as a complex. Furthermore, other standard means of administering peptides to cells can be utilized.

By "linked" as used herein is meant that the CARD peptide is associated with the signal peptide in such a manner that when the signal peptide crosses the cell membrane, the is also imported across the cell membrane. Examples of such means of linking include (1) the signal peptide can be linked by a peptide bond, i.e., the two peptides can be synthesized contiguously; (2) the signal peptide can be linked to the CARD peptide by a peptide bond or by a non-peptide covalent bond (such as conjugating a signal peptide to a CARD peptide with a crosslinking reagent); (3) the CARD peptide and the signal peptide can be joined by charge-association between the negatively-charged amino acids in the CARD peptide and the positively-charged amino acids in the peptide; (4) chemical ligation methods can be employed to create a covalent bond between the carboxy-terminal amino acid of the signal peptide and the CARD peptide. Methods (1) and (2) are typically preferred.

Examples of method (1) are shown below wherein a peptide is synthesized, by standard means known in the art,[24a,25a] that contains, in linear order from the amino-terminal end, a signal peptide sequence and a CARD peptide. Such a peptide could also be produced through recombinant DNA techniques, expressed from a recombinant construct encoding the above-described amino acids to create the peptide.[28a]

For method (2), either a peptide bond, as above, can be utilized or a non-peptide covalent bond can be used to link the signal peptide with the CARD peptide. This non-peptide covalent bond can be formed by methods standard in the art, such as by conjugating the signal peptide to the CARD peptide via a crosslinking reagent, for example, glutaraldehyde. Such methods are standard in the art.[29a] For method (3) the peptide can simply be mixed with the signal peptide and thus allowed to associate. These methods are performed in the same manner as association of peptides with cationic liposomes.[32a-34a] Such methods are standard in the art.

For method (4), standard chemical ligation methods, such as using chemical crosslinkers interacting with the carboxy-terminal amino acid of the signal peptide, can be utilized. Such methods are standard in the art (*see*, e.g., Goodfriend,[31a] which uses water-soluble carbodiimide as a ligating reagent) and can readily be performed to link the carboxy terminal end of the signal peptide to any selected CARD peptide.

The present invention provides a peptide comprising a cell adhesion regulatory domain of a β subunit of an integrin. The β subunit can be β_1. The β subunit can comprise amino acids 788-803 of β_1 subunit (SEQ ID NO:6). The β subunit can be β_3. The β subunit can comprise amino acids 747-762 of β_3 subunit (SEQ ID NO:2). The β subunit can be β_2. The β subunit can comprise amino acids 724-769 of β_2 subunit (SEQ ID NO:18). The present invention also provides a peptide comprising a cell adhesion regulatory domain of an α subunit of an integrin. The α subunit can be α_{IIb} subunit. The α subunit can comprise amino acids 989-1008 of α_{IIb} subunit (SEQ ID NO:10).

The invention further provides a chimeric peptide comprising an importation-competent peptide linked at the N-terminus to a cell adhesion regulatory domain of an adhesion receptor, adhesion molecule or counter receptor. An importation-competent peptide can be selected from any sequence that facilitates transport of proteins across a cellular membrane, such as signal peptides, hydrophobic regions of peptides, and helical structures in homeobox proteins. The invention specifically provides a chimeric peptide

comprising a signal peptide linked at the N-terminus to a cell adhesion regulatory domain of a β subunit of an integrin. The signal peptide can be the hydrophobic region (h-region) of the signal peptide sequence of human integrin β_3 : (SEQ ID NO:1), for example, or any other signal peptide or other hydrophobic sequences, as known in the art. The β subunit can be β_1. The peptide can comprise the signal peptide sequence of human integrin β_3 linked at the N-terminus to amino acids 788-803 of β_1 subunit (SEQ ID NO:12). The β subunit can be β_3. The peptide can comprise the signal peptide sequence of human integrin β_3 linked at the N-terminus to amino acids 747-762 of β_3 subunit (SEQ ID NO:11). The subunit β_3 can have a Ser^{752} to Ala^{752} substitution : YKEATATFTNITYRGT (SEQ ID NO:15). A chimeric peptide comprising the subunit β_3 can have a Ser^{752} to Ala^{752} substitution: VTVLALGALAGVGVGYKEATATFTNITYRGT (SEQ ID NO:16). The β subunit can be β_2. The chimeric peptide can comprise the signal peptide sequence of human integrin β_3 linked at the N-terminus to amino acids 724-769 of β_2 subunit (SEQ ID NO:18). Other substitutions can be determined following the methods set forth herein to determine substituted peptides that can be used to disrupt cellular adhesion.

The invention also specifically provides a chimeric peptide comprising a signal peptide linked at the N-terminus to a cell adhesion regulatory domain of an α subunit of an integrin. For example the α subunit can be α_{IIb}. The cell adhesion regulatory domain of an α subunit of an integrin can be linked to any importation-competent peptide, as exemplified herein by a signal peptide from integrin subunit β_3. The cell adhesion regulatory domain of an α_{IIb} subunit of an integrin can be the amino acid set forth in SEQ ID NO:10. A chimeric peptide can comprise the signal peptide sequence of human integrin β_3 linked at the N-terminus to amino acids 989-1008 of α_{IIb} subunit (SEQ ID NO:10), such as the chimeric peptide set forth in SEQ ID NO: 14.

The peptides of this invention can be made by any of several standard methods, such as chemical synthesis or by constructing recombinant molecules encoding the

chimeric protein, expressing and isolating the expressed chimeric protein, in a cell or in a cell-free system.

Statement concerning utility

The present invention can be used in any method and for any treatment in which inhibition of cellular adhesion can be beneficial. For example, the method can be used to prevent adhesion of leukemia cells. The method can be utilized to prevent metastatic growth of any tumors, since adhesion is required to form tumor masses. Since cancerous cells in the blood adhere to the walls of lymph nodes at very early stages of metastasis, this method can be particularly effective in cancer treatment, prevention and reduction. Additionally, the present method can be utilized to reduce or prevent inflammatory response by preventing adhesion of such cells. Furthermore, the present method can be directed to inhibiting adhesion of platelets to reduce or prevent blood clotting, and thus reduce or prevent conditions such as the progression of thrombosis and atherosclerosis. The present method can also be used to prevent adhesion of granulocytes, of endothelial cells, etc., and thus can be used to treat heart conditions, atherosclerosis, etc.

The present invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

EXAMPLES

Synthetic Peptides, Antibodies, and Cell Lines

Peptides were synthesized by a step-wise, solid-phase peptide synthesis method and purified by C_{18} reverse phase high performance liquid chromatography (HPLC) (10). As depicted in Figure 1, overlapping peptides encompassing the entire integrin β_3 cytoplasmic sequence (SEQ ID NO: 19) (6,7) represent residues 722-737 (peptide β_3-3), 735-750 (peptide β_3-2) (SEQ ID NO:4), 747-762 (peptide β_3-1) (SEQ ID NO:2), and 742-755 (peptide β_3-4) (SEQ ID NO:5). The cell-permeable peptides were

designed (10) by using the hydrophobic region Val-Thr-Val-Leu-Ala-Leu-Gly-Ala-Leu-Ala-Gly-Val-Gly-Val-Gly (h-region) (SEQ ID NO:1) of the signal peptide sequence of human integrin β_3 (6, 7) followed by the sequences of the cytoplasmic segments listed above. We also synthesized a cell-permeable peptide representing residues 788-803 of integrin β_1 (SEQ ID NO:6; Figure 1) (15,16). The molecular weights of the purified peptides were verified by mass spectrometry analysis and their composition and concentration by amino acid analysis. Polyclonal antibodies against the integrins β_3 and β_1 peptides without the hydrophobic region sequence were raised in rabbits immunized with a given peptide conjugated to keyhole limpet hemocyanin. The antibodies were monospecific for the respective β_3 peptides, as measured by ELISA. In addition, anti-integrin β_1 peptide antibody did not react with integrin β_3 peptides or with integrin $\alpha_{IIb}\beta_3$ heterodimer. Polyclonal anti-human integrin $\alpha_{IIb}\beta_3$ (Glycoprotein IIb-IIIa) antibodies were raised in rabbits using purified Glycoprotein IIb-IIIa (17). Anti-integrin β_1 monoclonal antibody (clone P4 C10) was obtained commercially from GIBCO BRL. The HEL cell line (11, 12) was obtained from Dr.Thalia Papayannopoulou, University of Washington, Seattle, Washington. Human endothelial cell line ECV304 (14) was obtained from Dr. Tom Maciag, American Red Cross Holland Laboratories, Rockville, Maryland, and human foreskin fibroblast cell line (18) was provided by Dr. Graham Carpenter, Vanderbilt University, Nashville, Tennessee.

Cell Adhesion Assay to Measure the Functional Effect of Cell-Permeable Peptides

Microtiter plates (96-well, Immulon-2, Dynatech) were coated with purified human fibrinogen (19) at 1.25 micrograms (μg)/milliliter (ml) and kept overnight at 4°C, washed with phosphate buffered saline (PBS), and incubated for 60 minutes at 37°C with 1% bovine serum albumin (BSA) to block non-specific sites. To measure the effect of the peptides on cell adhesion, HEL, ECV 304 cells or human fibroblasts (10^5 cells/well) were incubated with the indicated concentration of peptide at room temperature for 30 minutes in RPMI/10% serum, and centrifuged at 180 g. The peptide-containing supernatant was removed and cells were resuspended in RPMI/10% serum. PMA (10 nM) was added to only HEL cells, and cells were plated on fibrinogen-coated microtiter plates. Adhesion of human fibroblasts (HF)

was studied on fibrinogen-free plates. After incubation at 37°C for 120 minutes (HEL cells) and 240 minutes (ECV 304 and HF cell lines), the plates were washed 3 times with PBS and adherent cells were quantitated by cellular acid phosphatase assay (8). This assay measured acid phosphatase in ECV 304 cells although it was reported not detectable by a less sensitive immunochemistry technique (14). Per cent of inhibition of cell adhesion was determined after subtracting a background value obtained in ELISA. The effect of anti-integrin $\alpha_{IIb}\beta_3$ and anti-integrin β_1 antibodies on adhesion of HEL, ECV 304, and HF cell lines was tested by incubating cells with antibodies for 30 minutes at room temperature and then plating cells (10^5cell/well) and incubating for 4 hours at 37°C. After rinsing, adherent cells were quantified as above.

Detachment of adherent cells was analyzed by a modified procedure (20) using fibrinogen-coated microtiter plates, seeded with PMA-stimulated HEL cells or ECV 304 cells (10^5 cells/well). After incubation with tested peptides for 4 hours at 37°C, wells were rinsed and adherent cells were quantitated as described above.

Cell-Permeable Peptide Import Detection

Import of cell-permeable peptides was analyzed by confocal laser scanning microscopy of cells cytocentrifuged onto glass slides. Adherent cells were fixed with 3.5% paraformaldehyde, permeabilized with 0.25% Triton X-100, and reacted with respective monospecific antipeptide antisera for 1 hour at room temperature. Intracellular peptide-antibody complex was detected with rhodamine-conjugated anti-rabbit IgG (Kirkgaard & Perry). A Leitz confocal laser scanning microscope system was used with a 100X oil immersion lens as previously described (10). Alternatively, the import of peptides was quantitated by a Cell Enzyme-Linked Immunosorbent assay (Cell ELISA). Briefly, cells incubated with cell-permeable peptides (3-30μM) were washed, suspended in fresh medium and allowed to adhere to microtiter plates. After fixation and permeabilization, the cells were treated with monospecific antipeptide antibodies. Intracellular peptide-antibody complexes were detected with anti-rabbit IgG conjugated with alkaline phosphatase, and quantitated in ELISA.

Structure-Function Analysis of the Integrin β_3 Cytoplasmic Tail in HEL and ECV 304 Cells Using Cell-Permeable Peptides

For structure-function analysis of the cytoplasmic tail of integrin β_3, we synthesized four overlapping peptide analogs as specified in Figure 1. These peptides had no measurable effect on adhesion of PMA-stimulated HEL cells or ECV 304 cells to immobilized fibrinogen (Figure 2). However, when these peptides were rendered cell-permeable (10) through the addition of the hydrophobic (h) region sequence derived from the integrin β_3 signal peptide, they entered the cells and selectively exerted an inhibitory effect on cell adhesion to immobilized fibrinogen (Figure 2). Cell-permeable peptides were not cytotoxic within the concentrations used ($\leq$200μM), as determined by Trypan Blue exclusion.

The cell-permeable peptide β_3-1S, carrying the residues 747-762 of the β_3 cytoplasmic tail (SEQ ID NO:11), almost completely blocked the adhesion of both cell types to immobilized fibrinogen. In contrast, the cell-permeable peptides β_3-2S, β_3-3S, and β_3-4S were without measurable effect on cell adhesion. This structure-function analysis with cell-permeable peptides from the integrin β_3 cytoplasmic tail indicates that carboxy-terminal residues 747-762 (SEQ ID NO:11) constitute a functionally important sequence of the integrin β_3 cytoplasmic tail in two different cell types representing megakaryocytic and endothelial lineages. None of the tested peptides induced detachment of PMA-stimulated HEL cells when added 30 minutes after they were adherent to immobilized fibrinogen. Likewise, the tested peptides did not induce detachment of established monolayers of ECV 304 cells. All cell-permeable peptides were equally imported to the cytoplasm of HEL cells, as verified by confocal laser scanning microscopy following immunofluorescent staining with a peptide-specific antibody (Figure 3) and by quantitative analysis of imported peptides in cell ELISA of HEL and ECV 304 cells.

Inhibition of Cell Adhesion by Cell-Permeable Peptides is Integrin-Specific and Concentration-Dependent

Using integrin-specific antibodies, we determined that adhesion of HEL and ECV 304 cells to immobilized fibrinogen was mediated by integrin β_3 heterodimers

because anti-human integrin $\alpha_{IIb}\beta_3$ polyclonal antibody completely inhibited cell adhesion, while anti-human integrin β_1 antibody was without effect. On the other hand, adhesion of human fibroblasts to plastic was mediated by integrin β_1 heterodimers because anti-integrin β_1 inhibited adhesion, whereas anti-integrin $\alpha_{IIb}\beta_3$ antibody was without effect. Consistent with these results, cell-permeable peptide β_3-1S representing residues 747-762 of the cytoplasmic domain of integrin β_3 (SEQ ID NO:11) inhibited adhesion of HEL and ECV 304 cells to immobilized fibrinogen. (Fig. 4A and 4B). The cell-permeable β_1-1S peptide representing residue 788-803 of the cytoplasmic domain of integrin β_1 (SEQ ID NO:12) (15, 16) was non-inhibitory toward adhesion of HEL and ECV 304 cells to immobilized fibrinogen (Fig. 4A and Fig. 4B). On the other hand, adhesion of human fibroblasts to plastic mediated by integrin β_1 heterodimers was inhibited (75%) by cell-permeable β_1-1S peptide (200 μM), whereas cell-permeable β_3-1S peptide was inactive (Fig. 4C). These peptides were equally imported to HF cells as verified by cell ELISA (results not shown). In addition to the integrin-specific effects, the dose-response analysis indicates that the extracellular β_3-1S peptide concentrations required for 50% inhibition (EC_{50}) were 60μM and 55μM for HEL and ECV 304 cells, respectively. The EC_{50} of the cell permeable β_1-1S peptide in HF was 115μM. Because approximately 4% of cell-permeable peptide added to cells can be detected intracellularly (10) we estimate that intracellular peptide concentration causing 50% inhibition varies between 1-4μM. The cytoplasmic domains of human integrin β_1 and β_3 appear to be structurally similar (15, 16) as seven out of 16 residues in integrin β_1 segment (788-803) are identical with a corresponding sequence 747-762 of integrin β_3 (Figure 1). Since integrin β_3-mediated cell adhesion is inhibited from within by integrin β_3 peptide and integrin β_1- mediated adhesion is inhibited by integrin β_1 peptide, this pattern of inhibition indicates that regulation of the adhesive function of the integrin β_3 heterodimers in HEL and ECV 304 cells and integrin β_1 heterodimers in human fibroblasts follows an integrin-specific mechanism.

Cell-Permeable Mutant Peptides Identify Key Residues Involved in Regulation of Cell Adhesion

A "loss of function" point mutation $Ser^{752}Pro$ in the cytoplasmic segment of integrin β_3 is responsible for a life-long bleeding tendency and the abnormal adhesive function of integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) expressed in platelets of a Glanzmann thrombasthenia patient (5). This mutation lies in the functionally important segment of the integrin β_3 cytoplasmic tail identified in our experiments. Therefore, the question arises whether a $Ser^{752}Pro$ substitution in the functionally active cell-permeable peptide β_3-1S will result in a loss of its inhibitory function. Indeed, when the β_3-1S peptide had a $Ser^{752}\rightarrow Pro^{752}$ substitution, it lost inhibitory potency in a HEL and ECV 304 cell adhesion assay (Figure 5). To discern whether the $Ser^{752}Pro$ mutation is responsible for loss of function due to the lack of a potential phosphorylation site or due to the possible disruption by proline of the secondary structure of the β_3 integrin cytoplasmic tail, a second cell-permeable peptide with a $Ser^{752}Ala$ mutation (SEQ ID NO:15) was tested. This mutant peptide inhibited HEL and ECV 304 cell adhesion similarly to its wild-type β_3-1S analog (Figure 5). Thus, a proline-imposed effect on the secondary structure of the integrin β_3 cytoplasmic tail, rather than the loss of a potential phosphorylation site, can account for the observed differences. On the other hand, two tyrosine mutations in β_3-1S peptide involving conservative replacements $Tyr^{747}Phe$ and/or $Tyr^{759}Phe$ resulted in the loss of inhibitory function of β_3-1S peptide (Fig.6). Tyrosines 747 and 759 are therefore critically important for the inhibitory activity of the cell-permeable β_3-1S peptide. They constitute a functionally active tandem required for regulating the adhesive function of integrin β_3 in two different cell types. The role of phosphorylation of Tyr^{747} and Tyr^{759} in the function of β_3-1S peptide remains to be determined.

The results presented here indicate that the sequence 747-762 in integrin β_3 cytoplasmic tail (SEQ ID NO:2) constitutes the Cell Adhesion Regulatory Domain (CARD). The CARD of β_1 is found in amino acids 788-803 (SEQ ID NO:6). The sequence 724-769 in integrin β_2 cytoplasmic tail (SEQ ID Nos:9, 8, 7) contains an inhibitory peptide of β_2. The sequence 989-1008 in integrin α_{IIb} cytoplasmic tail (SEQ ID NO:10) represents the CARD of α_{IIb}. Although other motifs such as the conserved membrane-proximal short sequences present in the cytoplasmic "hinge" of integrins α_{IIb} and β_3 may also be involved (21), our structure-function analysis with a panel of cell-

permeable peptides suggests that CARD plays a pivotal role in the cell adhesive function of integrin β_3. Moreover, a homologous segment in integrin β_1 appears to regulate adhesion of human fibroblasts mediated by integrin β_1 heterodimers. Thus CARD is involved in integrin-specific regulation of cell adhesion. Our results transcend previous experiments with transiently expressed $\alpha_{IIb}\beta_3$ in heterologous CHO cells (9). In that study, the entire β_3 cytoplasmic tail was truncated, thereby abolishing cell spreading and adhesion mediated by $\alpha_{IIb}\beta_3$ recruited to focal adhesions. Regulation of binding of monoclonal antibody PAC1 to CHO cells doubly transfected with integrin $\alpha_{IIb}\alpha_{6B}$ and β_3 or β_1 chimeras appears to involve the NPXY motif (22). This motif is found in many integrin β subunits and is implicated in integrin localization in focal adhesions (23), in cleavage of the integrin β_3 cytoplasmic tail by calpain (24), in internalization of other membrane receptors (25); and in binding of a novel phosphotyrosine-binding (PTB) domain (26). However, two distinct cell-permeable peptides β_3-2S and β_3-4S that contained the ^{744}NPLY^{747} motif did not inhibit adhesion of HEL and ECV304 cells in our experimental system. This finding together with results of Tyr^{747}Phe and Tyr^{759}Phe mutations within the β_3-1S peptide support the proposal that the two tyrosines (747 and 759), acting in tandem within CARD, are essential for regulation of the adhesive function of integrin β_3. Similarly spaced tyrosines play a role in the interaction of T and B cell antigen receptor cytoplasmic tails (27). The functionally active β_3-1S peptide imported to HEL and ECV 304 cells can exert its inhibitory effect by interacting with α_{IIb} or α_v integrins, respectively. Alternatively, the β_3-1S peptide can interact with other CARD-recognizing cytoplasmic proteins, e.g. β_3 endonexin (28). The identity of cytoplasmic protein(s) interacting with CARD remains to be established.

Cell-Permeable Peptide Turnover Is Regulated by the Multidrug Resistance Pump (P-Glycoprotein).

The membrane P-glycoprotein (P170; mdr1 gene product) is an ATP-hydrolyzing transmembrane pump. It is responsible for multidrug resistant phenotype by its capacity to prevent intracellular accumulation of unrelated chemotherapeutic drugs, usually of hydrophobic nature (57). It is possible to reverse the function of the multidrug resistance pump by three categories of compounds: chemotherapeutic drug

analogs, calcium channel antagonists (*e.g.*, verapamil), immunosuppressive cyclic peptides (*e.g.*, cyclosporins) and calmodulin inhibitors **(57)**. They reverse MDR by inhibiting drug efflux and therefore increasing accumulation of drug in MDR cells. Similarly, drug accumulation is observed in mdr gene knockout mice **(58)**. To test our hypothesis that MDR pump is involved in regulation of intracellular level of cell-permeable peptides imported to HEL cells we used MDR pump-reversing agent, verapamil. Cell-permeable peptide β_3-1S known to inhibit adhesion of phorbol ester-stimulated HEL cells to immobilized fibrinogen was assayed in the absence and presence of verapamil. The extracellular concentration of β_3-1S peptide necessary to cause 50% inhibition of adhesion (EC_{50}) in HEL cells in 80μM. When cells were pretreated with verapamil (1 μM) the EC_{50} of β_3-1S peptide was reduced to 15μM. This means that effective intracellular concentration of cell-permeable β_3-1S peptide was 6 times higher when MDR pump was reversed by verapamil (Fig. 8).

HEL cells treated with verapamil alone had unimpaired adhesion and there is no indication the verapamil inhibited cell-permeable peptide import to cells. These experiments indicate that the MDR pump is involved in regulation of cell-permeable peptide efflux. When the efflux is blocked, cell-permeable peptide accumulates in HEL cells thereby exerting more pronounced inhibitory effect on intracellular protein-protein interactions involved in regulation of adhesive function of integrins β_3. Thus, cell-permeable peptides can move in and out of the cells. These results are consistent with reports from other laboratories that MDR pump is involved in binding a number of intracellular peptides **(59), (60).**

In summary, structure-function analysis of the intracellular segment of integrins using cell-permeable peptides pinpoints the Cell Adhesion Regulatory Domains. Inhibition of integrin $\alpha_{IIb}\beta_3$-mediated cell adhesion to immobilized fibrinogen by functionally active cell-permeable peptides is an alternative to pharmacologic blockade of the extracellular, ligand-binding domains of this integrin (29). Imported β_3 peptides compete with the endogenous integrin β_3 cytoplasmic tail to interrupt integrin-specific intracellular protein-protein interactions that engage the cytoplasmic "business end" of

integrin β_3. Moreover, CARD representing integrin β_1 cytoplasmic segment can inhibit adhesion of human fibroblasts in integrin specific manner. This approach offers a unique opportunity to modulate the adhesive functions of cellular integrins "from within." Use of the import of a CARD peptide into cells is enhanced by also administering a composition comprising a compound that can block the export of peptides from a particular cell, such as a calcium channel blocker.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Val Thr Val Leu Ala Leu Gly Ala Leu Ala Gly Val Gly Val Gly
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Tyr Lys Glu Ala Thr Ser Thr Phe Thr Asn Ile Thr Tyr Arg Gly Thr
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ala Arg Ala Lys Trp Asp Thr Ala Asn Asn Pro Lys Tyr Lys Glu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

His Asp Arg Lys Glu Phe Ala Lys Phe Glu Glu Glu Arg Ala Arg Ala
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ala Asn Asn Pro Leu Tyr Lys Glu Ala Thr Ser Thr Phe Thr
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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Tyr Lys Ser Ala Val Thr Thr Val Val Asn Pro Lys Tyr Glu Gly Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Lys Ser Ala Thr Thr Thr Val Met Asn Pro Lys Phe Ala Glu Ser
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Lys Glu Lys Leu Lys Ser Gln Trp Asn Asn Asp Asn Pro Leu Phe Lys
1 5 10 15
Ser

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Lys Ala Leu Ile His Leu Ser Asp Leu Arg Glu Tyr Arg Arg Phe Glu
1 5 10 15
Lys Glu

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Lys Val Gly Phe Phe Lys Arg Asn Arg Pro Pro Leu Glu Glu Asp Asp
1 5 10 15
Glu Glu Gly Glu
20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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 1               5                  10                  15
Lys Glu Ala Thr Ser Thr Phe Thr Asn Ile Thr Tyr Arg Gly Thr
            20                  25                  30
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(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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Val Thr Val Leu Ala Leu Gly Ala Leu Ala Gly Val Gly Val Gly Tyr
 1               5                  10                  15
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            20                  25                  30
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(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Val Thr Val Leu Ala Leu Gly Ala Leu Ala Gly Val Gly Val Gly Lys
 1               5                  10                  15
```

Ser Ala Thr Thr Thr Val Met Asn Pro Lys Phe Ala Glu Ser
20 25 30

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Val Thr Val Leu Ala Leu Gly Ala Leu Ala Gly Val Gly Val Gly Lys
1 5 10 15
Val Gly Phe Phe Lys Arg Asn Arg Pro Pro Leu Glu Glu Asp Asp Glu
20 25 30
Glu Gly Glu
35

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Tyr Lys Glu Ala Thr Ala Thr Phe Thr Asn Ile Thr Tyr Arg Gly Thr
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Val Thr Val Leu Ala Leu Gly Ala Leu Ala Gly Val Gly Val Gly Tyr
1 5 10 15
Lys Glu Ala Thr Ala Thr Phe Thr Asn Ile Thr Tyr Arg Gly Thr
20 25 30

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Lys Ala Leu Ile His Leu Ser Asp Leu Arg Glu Tyr Arg Arg Phe Glu
1 5 10 15
Lys Glu Lys Leu Lys Ser Gln Trp Asn Asn Asp Asn Pro Leu Phe Lys

20 25 30
Ser Ala Thr Thr Thr Val Met Asn Pro Lys Phe Ala Glu Ser
35 40 45

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

His Asp Arg Lys Glu Phe Ala Lys Phe Glu Glu Glu Arg Ala Arg Ala
1 5 10 15
Lys Trp Asp Thr Ala Asn Asn Pro Lys Tyr Lys Glu Ala Thr Ser Thr
20 25 30
Phe Thr Asn Ile Thr Tyr Arg Gly Thr
35 40

SEQ ID NO: 20: peptide
LFKSATTTVMNPKFAES

SEQ ID NO: 21: peptide
KEKLKSQWNNDNPLF

What is claimed is:

1. A method of inhibiting adhesion of a cell comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by the cell.

2. The method of claim 1, wherein the adhesion receptor is an integrin.

3. The method of claim 2, wherein the subunit is a β subunit of an integrin.

4. The method of claim 3, wherein the β subunit is β_3.

5. The method of claim 3, wherein the β subunit is β_2.

6. The method of claim 3, wherein the β subunit is β_1.

7. The method of claim 2, wherein the subunit is an α subunit of an integrin.

8. The method of claim 7, wherein the α subunit is α_{IIb}.

9. The method of claim 7, wherein the α subunit is α_L.

10. The method of claim 7, wherein the α subunit is α_M.

11. The method of claim 7, wherein the α subunit is α_X.

12. The method of claim 1, wherein the adhesion receptor is a selectin.

13. The method of claim 12, wherein the selectin is L-selectin.

14. The method of claim 12, wherein the selectin is E-selectin.

15. The method of claim 12, wherein the selectin is P-selectin.

16. The method of claim 1, wherein the adhesion receptor is a cadhesin.

17. The method of claim 16, wherein the cadhesin is E-cadhesin.

18. The method of claim 16, wherein the cadhesin is N-cadhesin.

19. The method of claim 16, wherein the cadhesin is P-cadhesin.

20. The method of claim 1, wherein the counter receptor is an ICAM.

21. The method of claim 20, wherein the ICAM is ICAM-1.

22. The method of claim 20, wherein the ICAM is ICAM-2.

23. The method of claim 20, wherein the ICAM is ICAM-3.

24. The method of claim 1, wherein the cell is a cancer cell.

25. The method of claim 1, wherein the cell is a fibroblast and the adhesion receptor is an integrin comprising subunit β_1.

26. The method of claim 1, wherein the peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor is transferred into the cell by contacting the cell with a chimeric peptide comprising an importation-competent signal peptide at the N-terminus of the chimeric peptide and the cell adhesion regulatory domain, thereby transferring the peptide and inhibiting adhesion of the cell.

27. The method of claim 26, wherein the cell is contacted with the chimeric peptide and a compound that blocks a cellular membrane channel exporting peptides and drugs to outside the cell.

28. The method of claim 27, wherein the compound is verapamil.

29. The method of claim 27, wherein the compound is a cyclosporin.

30. The method of claim 1, wherein the peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor is transferred into the cell by contacting the cell with a complex comprising the cell adhesion regulatory domain linked to an importation-competent signal peptide, thereby transferring the peptide and inhibiting adhesion of the cell.

31. A method of reducing or preventing an inflammatory response of a cell comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by the cell.

32. The method of claim 31, wherein the adhesion receptor is an integrin.

33. The method of claim 32, wherein the subunit is a β subunit of an integrin expressed by the cell.

34. The method of claim 32, wherein the subunit is an α subunit of an integrin expressed by the cell.

35. A method of reducing or preventing excessive proliferation of a fibroblast comprising transferring into the fibroblast a peptide comprising a cell adhesion regulatory domain of a β_1 subunit of an integrin receptor.

36. The method of claim 35, wherein the peptide comprises the amino acid sequence set forth in SEQ ID NO:6.

37. The method of claim 35, wherein the peptide comprises the amino acid sequence set forth in SEQ ID NO:12.

38. The method of claim 35, wherein the reduction or prevention is sufficient to effect wound healing.

39. A method of reducing or preventing blood clotting in a subject comprising contacting a platelet with a chimeric peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, thereby reducing or preventing platelet cell adhesion and reducing or preventing blood clotting in the subject.

40. The method of claim 39, wherein the β subunit is β_3.

41. The method of claim 39, wherein the peptide comprises the amino acid sequence set forth in SEQ ID NO:2.

42. A method of reducing or preventing platelet adhesion in extracorporeal circulation of a subject comprising contacting *ex vivo* during the extracorporeal circulation process a platelet from the subject with a chimeric peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, and replacing the platelet into the subject, thereby reducing or preventing platelet cell adhesion in the extracorporeal circulation.

43. A method of reducing or preventing platelet cell adhesion in a subject comprising contacting a platelet with a chimeric peptide comprising a signal peptide at the N-

terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, thereby reducing or preventing platelet cell adhesion in the subject.

44. A method of treating or preventing coronary and/ or vascular disease or conditions in a subject comprising administering to the subject a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by a vascular endothelial cell, thereby reducing or inhibiting attachment of leukocytes and platelets to an atherosclerotic lesion.

45. A method of reducing or preventing granulocyte adhesion in a subject comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by the granulocyte, thereby reducing or preventing adhesion of the granulocyte in the subject.

46. A method of preventing or reducing restenosis in the blood vessels of a subject, comprising administering to the subject comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by an endothelial cell of the blood vessel, a granulocyte or a platelet, thereby reducing or preventing adhesion of the endothelial cells, granulocyte, and/or platelet in the subject.

47. A method of treating adult respiratory distress syndrome in a subject comprising administering to the subject a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by a granulocyte of the subject, thereby preventing or reducing adhesion of a granulocyte to the endothelial lining of the microvasculature of the lungs and treating adult respiratory distress syndrome.

48. A peptide comprising a cell adhesion regulatory domain of a β subunit of an integrin.

49. The peptide of claim 48, wherein the β subunit is β_1.

50. The peptide of claim 49, wherein the β subunit comprises amino acids 788-803 of β_1 subunit.

51. The peptide of claim 48, wherein the β subunit is β_3.

52. The peptide of claim 51, wherein the β subunit comprises amino acids 747-762 of β_3 subunit.

53. The peptide of claim 48, wherein the β subunit is β_2.

54. The peptide of claim 53, wherein the β subunit comprises amino acids 724-769 of β_2 subunit.

55. The peptide of claim 53, wherein the β subunit has a Ser^{752} to Ala^{752} substitution.

56. A peptide comprising a cell adhesion regulatory domain of an α subunit of an integrin.

57. A peptide comprising a signal peptide linked at the N-terminus to a cell adhesion regulatory domain of a β subunit of an integrin.

58. A peptide comprising a signal peptide linked at the N-terminus to a cell adhesion regulatory domain of an α subunit of an integrin.

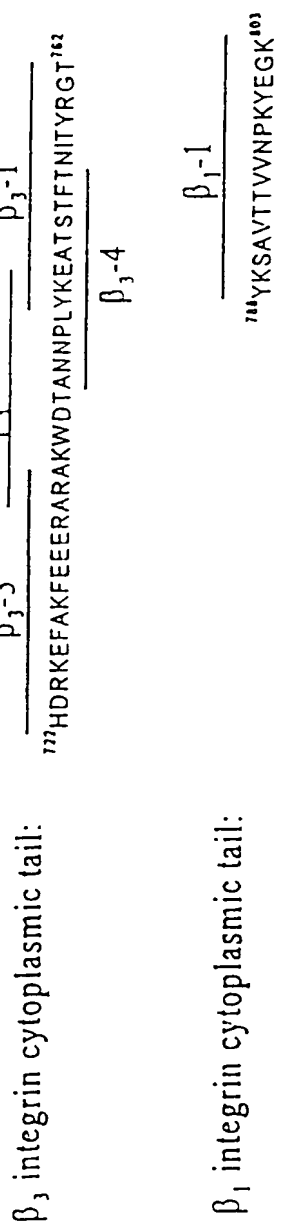

FIGURE 1

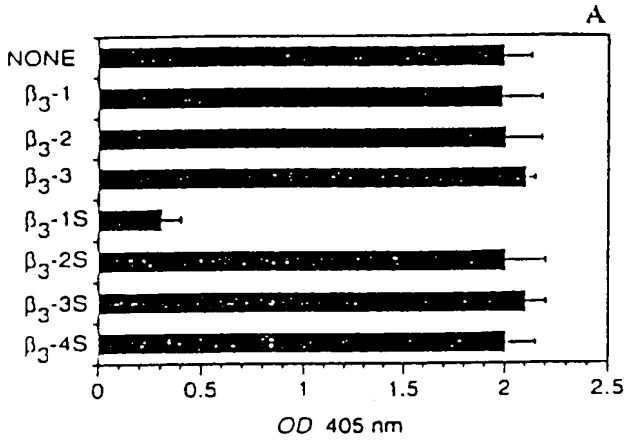

FIGURE 2A

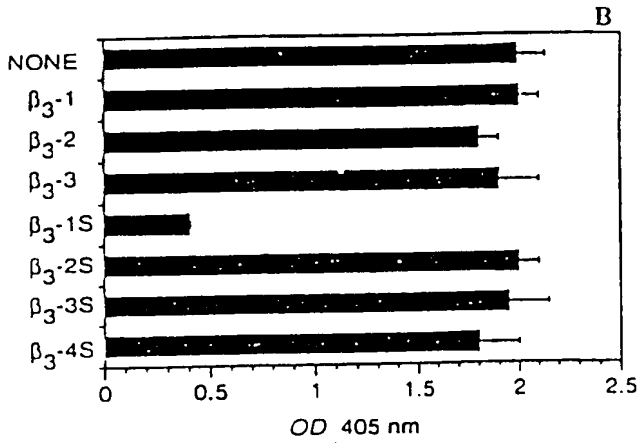

FIGURE 2B

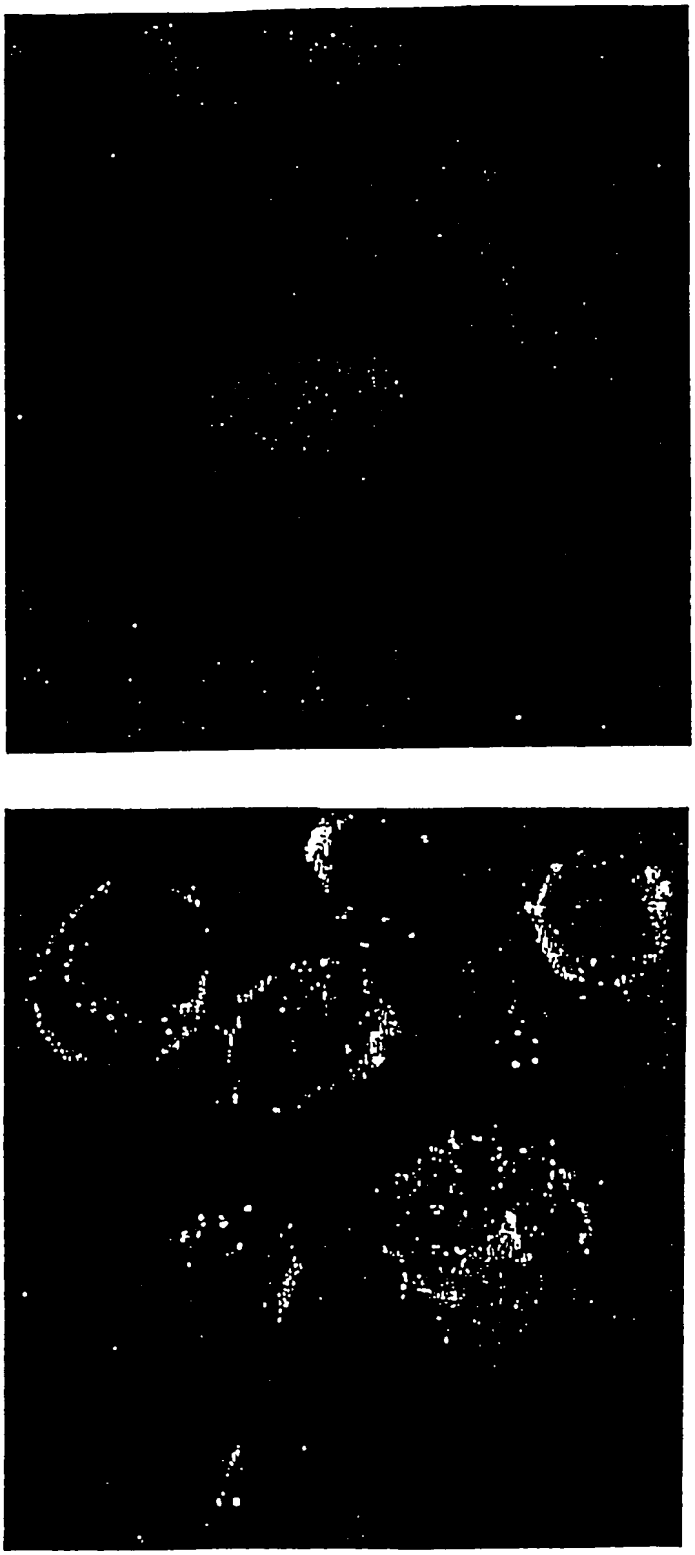

FIGURE 3

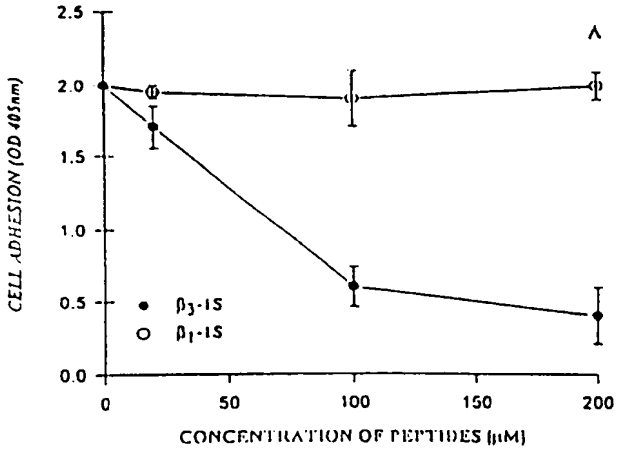

FIGURE 4A

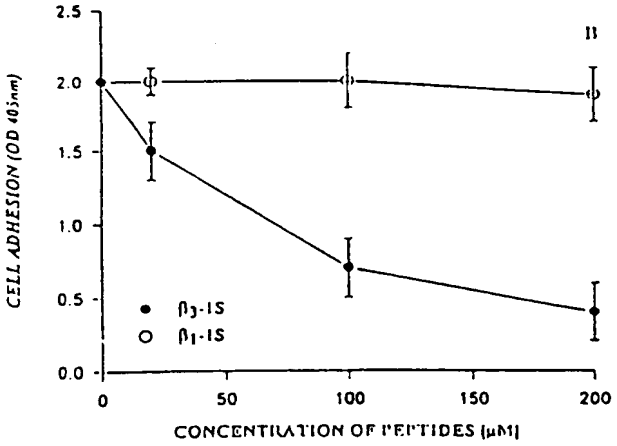

FIGURE 4B

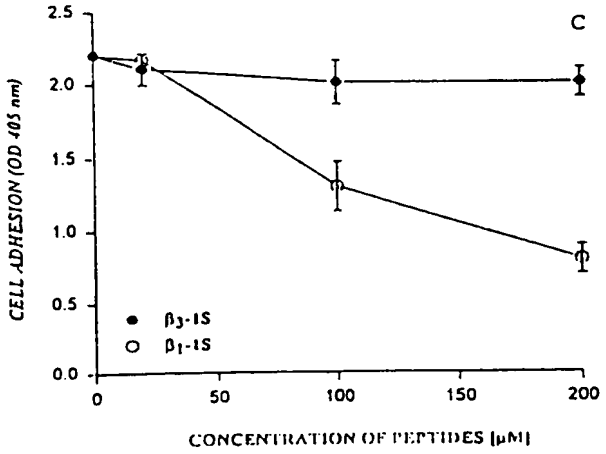

FIGURE 4C

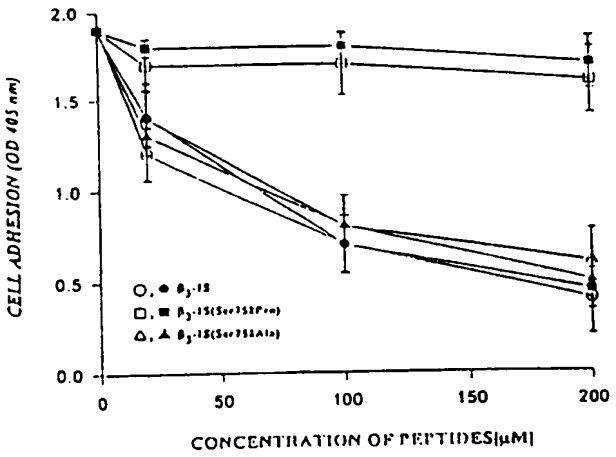

FIGURE 5

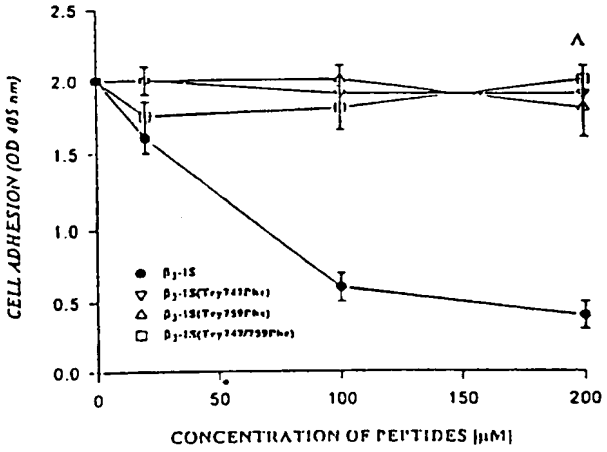

FIGURE 6A

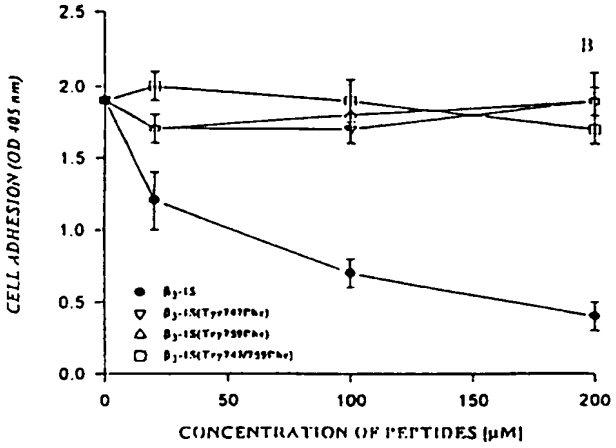

FIGURE 6B

DESIGN OF CELL-PERMEABLE PEPTIDES IMPORTING CYTOPLASMIC TAIL SEQUENCES OF β_3 INTEGRIN INTO HEL CELLS

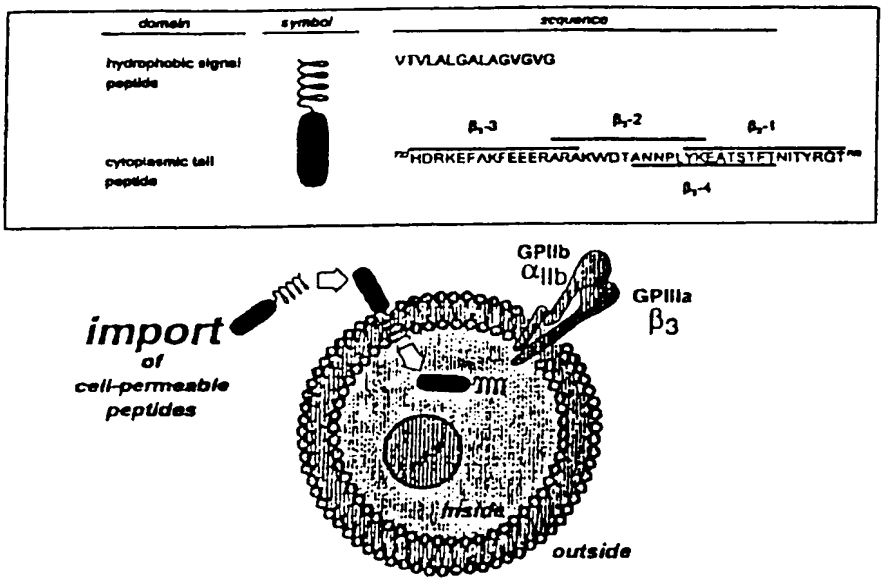

FIGURE 7

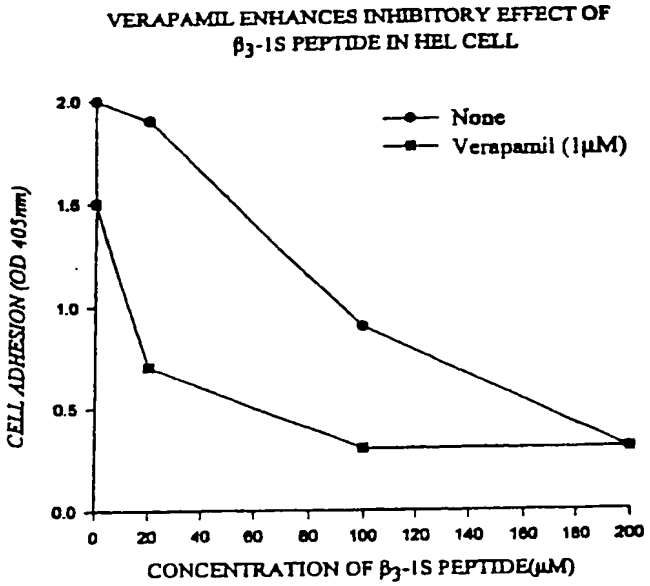

FIGURE 8

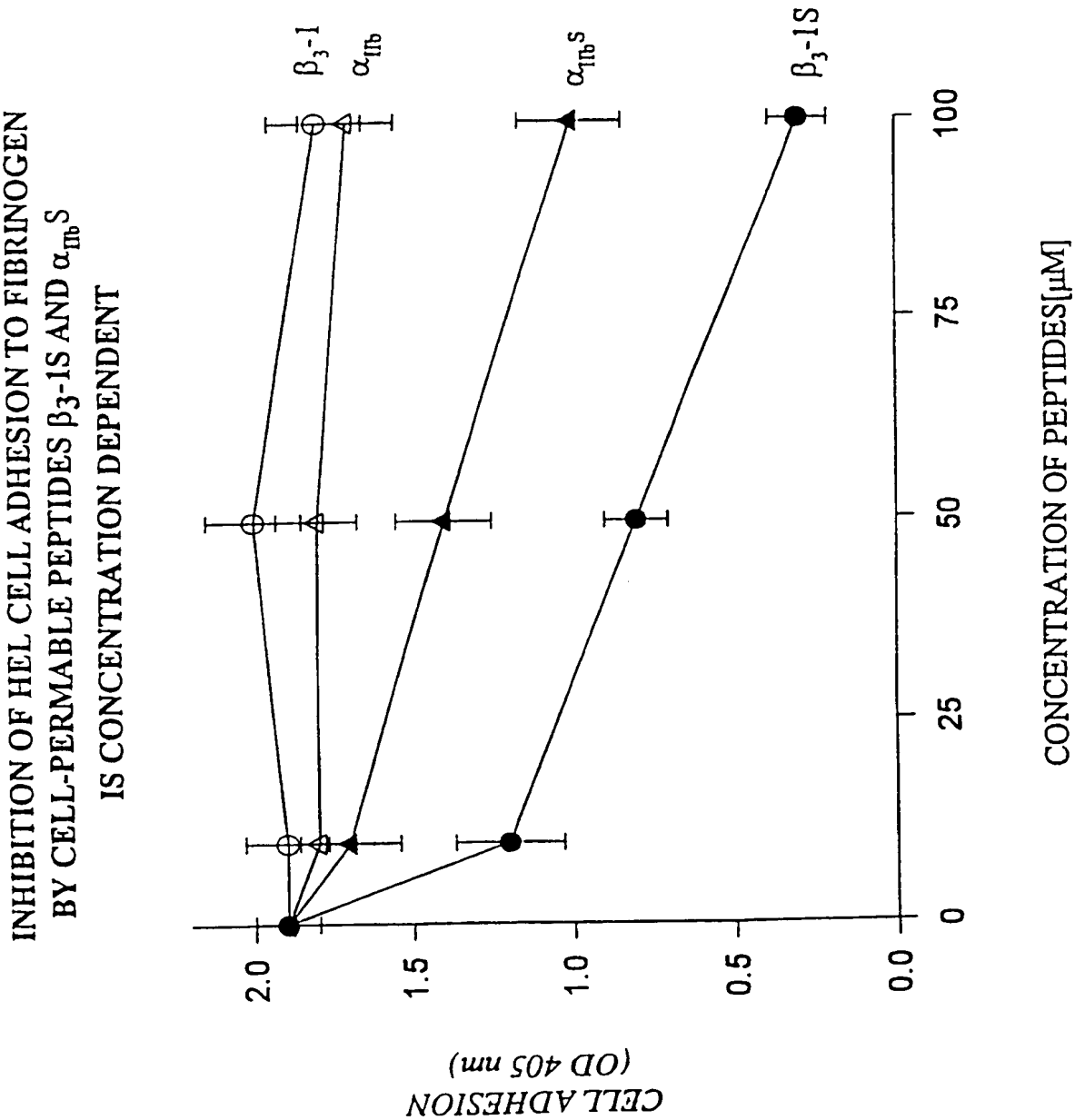

FIGURE 9

FIGURE 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18331

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/10, 38/16, 31/335, 31/135; C07K 7/08, 14/47

US CL :514/2, 12, 450, 646; 530/300, 324, 326

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 12, 450, 646; 530/300, 324, 326

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS/USPAT, STN/Medline, CaPlus
search terms: beta, integrin#, peptide#, cytoplasm?, chimer?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LAFLAMME, S.E. et al. Single Subunit Chimeric Integrins as Mimics and Inhibitors of Endogenous Integrin Functions in Receptor Localization, Cell Spreading and Migration, and Matrix Assembly. September 1994. Journal of Cell Biology, Volume 126, Number 5, pages 1287-1298, especially pages 1292-1295.	1-4, 6, 25, 35-38, 48-52, 57
X ----- Y	LUKASHEV, M.E. et al. Disruption of Integrin Function and Induction of Tyrosine Phosphorylation by the Autonomously Expressed Beta 1 Integrin Cytoplasmic Domain. 15 July 1994. Journal of Biological Chemistry. Volume 269, Number 28, pages 18311-18314, especially Fig. 3 and pages 18312-18314.	1-3, 6, 25, 35-38, 48-50 ----- 5, 31-33, 45-46, 53-54

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 DECEMBER 1997	Date of mailing of the international search report 27 JAN 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KAREN E.BROWN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18331

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,340,800 A (LIU et al) 23 August 1994, column 1, line 36 to column 3, line 15.	5, 31-33, 45-46, 53-54
P, X ------ P, Y	LIU, X.-Y. et al. Identification of a Functionally Important Sequence in the Cytoplasmic Tail of Integrin Beta 3 by Using Cell-Permeable Peptide Analogs. 15 October 1996. Proceedings of the National Academy of Science USA, Volume 93, pages 11819-11824, see entire document.	1-4, 6, 24-26, 31-33, 35-38, 48-52, 57 ----- 39-44, 46
Y	US 5,114,842 A (PLOW et al.) 19 May 1992, column 1, line 65 to column 2, line 52; and column 21, line 12 to column 22, line 42.	39-44, 46
A	LIN, Y.-Z. et al. Inhibition of Nuclear Translocation of Transcription Factor NF-Kappa B by a Synthetic Peptide Containing a Cell Membrane-Permeable Motif and Nuclear Localization Sequence. 16 June 1995. Journal of Biological Chemistry, Volume 270, Number 24, pages 14255-14258, see entire document.	1-6, 24-33, 35-55, 57
A	RUOSLAHTI, E. et al. Anchorage Dependence, Integrins and Apoptosis. 20 May 1994. Cell, Volume 77, pages 477-478, see entire document.	1-6, 24, 30, 35-38

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18331

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-6, 24-33, 35-55, 57

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18331

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:

Beta integrin subunits
Alpha integrin subunits
Selectins
Cadherins
ICAMs

The claims are deemed to correspond to the species listed above in the following manner:

Beta integrin subunits: claims 3-6, 25, 33, 35-43, 48-55, 57
Alpha integrin subunits: claims 7-11, 34, 56, 58
Selectins: claims 12-15
Cadherins: claims 16-19
ICAMs: claims 20-23

The following claims are generic: 1-2, 24, 26-32, 44-47

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons:

The invention recites a method of inhibiting cell adhesion by transferring a peptide comprising a cell adhesion regulatory domain derived from an adhesion protein into a cell. The special technical feature of the first species recited is the cell regulatory domain derived from a beta integrin subunit. This special technical feature is not shared with the species of the alpha integrin subunits, the selectins, the cadherins or the ICAMs because the cell regulatory domains of each of these species are completely different from the beta integrin subunit and from one another. The cell regulatory domain of each species are structurally different from each other and from the cell regulatory domain of the beta integrin subunits because each cell regulatory domain contains completely different amino acid sequences. Furthermore, each of these cell regulatory domains are functionally different from each other because the cell regulatory domains of each species interact with a different set of intracellular proteins in the claimed method. Therefore, since none of the other species' cell regulatory domains are structurally or functionally similar to that of the beta integrin subunits, these species do not share the same special technical feature with that of the beta integrin subunit. In addition, since none of the species' cell regulatory domains are structurally or functionally similar to each other's, these species also do not share a special technical feature with each other.

CORRECTED VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 38/10, 38/16, 31/335, 31/135, C07K 7/08, 14/47

A1

(11) International Publication Number: **WO 98/16241**

(43) International Publication Date: 23 April 1998 (23.04.98)

(21) International Application Number: PCT/US97/18331

(22) International Filing Date: 9 October 1997 (09.10.97)

(30) Priority Data:
60/028,420 15 October 1996 (15.10.96) US

(71) Applicant *(for all designated States except US)*: VANDERBILT UNIVERSITY [US/US]; 305 Kirkland Hall, Nashville, TN 37240 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: HAWIGER, Jack, J. [US/US]; 1818 Laurel Ridge Drive, Nashville, TN 37215 (US). TIMMONS, Sheila [US/US]; 4207 Belmont Park Terrace, Nashville, TN 37215 (US). LIU, Xue–Yan [CN/US]; 3000 Hillsboro Road #113, Nashville, TN 37215 (US).

(74) Agents: SELBY, Elizabeth et al.; Needle & Rosenberg, 127 Peachtree Street, N.E., Atlanta, GA 30303 (US).

(81) Designated States: AU, CA, US.

Published
With international search report.

(54) Title: METHOD OF DISRUPTING CELLULAR ADHESION

(57) Abstract

The present invention provides a method of inhibiting or disrupting cellular adhesion of a cell comprising transferring into the cell a polypeptide comprising a cell adhesion regulatory domain of an adhesion receptor or counter receptor expressed by the cell. In particular, the present invention provides a method of inhibiting or disrupting cellular adhesion of a cell comprising transferring into the cell a polypeptide comprising a cell adhesion regulatory domain of a subunit, i.e., the αsubunit or the βsubunit of an integrin expressed by the cell.

*(Referred to in PCT Gazette No. 26/1998, Section II)

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BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
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METHOD OF DISRUPTING CELLULAR ADHESION

This invention was made with government support under the National Institutes of Health Grants HL45994 and HL30647. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to methods of inhibiting cellular adhesion by interfering with the functioning of surface molecules participating in the cellular adhesion. In particular, it relates to transferring into a cell to be inhibited a cellular adhesion regulatory domain peptide of an adhesion receptor or counter receptor expressed by that cell, which transfer thereby disrupts adhesion of the cell.

Background Art

Integrins are major two-way signaling receptors responsible for the attachment of cells to the extracellular matrix and for cell-cell interactions that underlie developmental programming, immune responses, tumor metastasis, and progression of atherosclerosis and thrombosis. The latter involves formation of vasoocclusive platelet thrombi bridged by fibrinogen bound to integrin $\alpha_{IIb}\beta_3$. Integrins composed of non-identical α and β subunits recognize ligands through extracellular domains and transmit intracellular signals through cytoplasmic tails. Outside-in post-ligand binding functions, such as integrin recruitment to focal adhesions and cell spreading, also depend on integrin cytoplasmic segments (1-3). Signal-dependent binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$ (Glycoprotein IIb-IIIa complex) expressed on platelets provides the key mechanism for formation of hemostatic and vasoocclusive thrombi (4). Genetic defects in integrin $\alpha_{IIb}\beta_3$ are responsible for Glanzmann's thrombasthenia, a life-long bleeding tendency arising from the inability of human platelets to bind fibrinogen. Among many mutations responsible for integrin $\alpha_{IIb}\beta_3$ dysfunction in Glanzmann's thrombasthenia, a

point mutation $Ser^{752}Pro$ in the integrin β_3 cytoplasmic tail is of particular interest (5). This "loss of function" integrin β_3 mutation exemplifies the important role of the cytoplasmic segment of integrin β_3 in regulating the adhesive function of the extracellular domain. The cytoplasmic segment of integrin β_3 comprises 41 residues from 722 through 762 (6,7). Deletion of the entire β_3 cytoplasmic segment led to the loss of adhesive function of transiently transfected Chinese Hamster Ovary (CHO) cells (8,9). A structure-function analysis of the cytoplasmic segment of integrin β_3 is needed to pinpoint its regulatory sites. Such an analysis was conducted in the present invention using a non-invasive cellular import method based on cell-permeable properties of hydrophobic (h) region of a signal peptide sequence.

Signal peptide sequences,[1a] which share the common motif of hydrophobicity, mediate translocation of most intracellular secretory proteins across mammalian endoplasmic reticulum (ER) and prokaryotic plasma membranes through the putative protein-conducting channels.[2a-11a] Alternative models for secretory protein transport also support a role for the signal sequence in targeting proteins to membranes.[12a-15a]

Several types of signal sequence-mediated inside-out membrane translocation pathways have been proposed. The major model implies that the proteins are transported across membranes through a hydrophilic protein-conducting channel formed by a number of membrane proteins.[2a-11a] In eukaryotes, newly synthesized proteins in the cytoplasm are targeted to the ER membrane by signal sequences that are recognized generally by the signal recognition particle (SRP) and its ER membrane receptors. This targeting step is followed by the actual transfer of protein across the ER membrane and out of the cell through the putative protein-conducting channel (for recent reviews, see references 2a-5a). In bacteria, the transport of most proteins across the cytoplasmic membrane also requires a similar protein-conducting channel.[7a-11a] On the other hand, signal peptides can interact strongly with lipids, supporting the proposal that the transport of some secretory proteins across cellular membranes may occur directly through the lipid bilayer in the absence of any proteinaceous channels.[14a-15a]

The present invention provides a structure-function analysis of the cytoplasmic tail of cell surface receptors based on the cellular import of synthetic peptide analogs of this region. It was determined that a peptide carrying certain residues of a subunit of the receptor could be utilized to inhibit function of the receptor. Inhibition was found to be receptor-specific. Thus the present invention provides a surprising method of inhibiting receptor function that can be very specifically directed to inhibition of a specific receptor, and thus a specific cell and a specific disease or disease-producing condition to be treated. The present invention can be utilized for a wide variety of uses, such as inhibition of adhesion and proliferation of specific cells.

SUMMARY OF THE INVENTION

The present invention provides a method of inhibiting or disrupting cellular adhesion of a cell comprising transferring into the cell a polypeptide comprising a cell adhesion regulatory domain of an adhesion receptor or counter receptor expressed by the cell. In particular, the present invention provides a method of inhibiting or disrupting cellular adhesion of a cell comprising transferring into the cell a polypeptide comprising a cell adhesion regulatory domain of a subunit, *i.e.*, the α subunit or the β subunit of an integrin expressed by the cell.

Also provided is a method of reducing or preventing an inflammatory response of a cell comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by the cell.

The present invention further provides a method of reducing or preventing blood clotting in a subject comprising contacting a platelet with a chimeric peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, thereby reducing or preventing platelet cell adhesion and reducing or preventing blood clotting in the subject.

Further provided is a method of reducing or preventing platelet adhesion in extracorporeal circulation of a subject comprising contacting *ex vivo* during the extracorporeal circulation process a platelet from the subject with a chimeric peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, and replacing the platelet into the subject, thereby reducing or preventing platelet cell adhesion in the extracorporeal circulation.

The present invention additionally provides a method of reducing or preventing platelet cell adhesion in a subject comprising contacting a platelet with a chimeric

peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, thereby reducing or preventing platelet cell adhesion in the subject.

Also provided is a method of treating or preventing coronary and/ or vascular disease or conditions in a subject comprising administering to the subject a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by a vascular endothelial cell, thereby reducing or inhibiting attachment of leukocytes and platelets to an atherosclerotic lesion.

The present invention further provides a method of reducing or preventing granulocyte adhesion in a subject comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by the granulocyte, thereby reducing or preventing adhesion of the granulocyte in the subject.

Also provided is a method of preventing or reducing restenosis in the blood vessels of a subject, comprising administering to the subject comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by an endothelial cell of the blood vessel, a granulocyte or a platelet, thereby reducing or preventing adhesion of the endothelial cells, granulocyte, and/or platelet in the subject.

Further provided by the present inventio is a method of treating adult respiratory distress syndrome in a subject comprising administering to the subject a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by a granulocyte of the subject, thereby preventing or reducing adhesion of a granulocyte to the endothelial lining of the microvasculature of the lungs and treating adult respiratory distress syndrome.

Additionally provided is a peptide comprising a cell adhesion regulatory domain of a β subunit of an integrin. The present invention further provides a peptide comprising a cell adhesion regulatory domain of an α subunit of an integrin.

Also provided by the present invention is a peptide comprising a signal peptide linked at the N-terminus to a cell adhesion regulatory domain of a β subunit of an integrin. The present invention also provides a peptide comprising a signal peptide linked at the N-terminus to a cell adhesion regulatory domain of an α subunit of an integrin.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG 1. Sequence of overlapping peptides representing the integrin β_3 cytoplasmic tail (SEQ ID NO:19), including peptides β_3-1 (SEQ ID NO:2), β_3-2 (SEQ ID NO:3) and β_3-3 (SEQ ID NO:4) and of integrin β_1 cytoplasmic tail peptide homologous to β_3-1 peptide (SEQ ID NO:6) (all in single-letter amino acid code).

FIG 2. Effect of the integrin β_3 cytoplasmic tail peptides on adhesion of HEL (A) or ECV 304 (B) cells to immobilized fibrinogen. HEL cells were preincubated in the absence (NONE) and presence of 200 μM peptide. The bars labeled with β_3-3, β_3-2, and β_3-1 represent adhesion of cells treated with non-cell-permeable peptides encompassing the integrin β_3 cytoplasmic sequence containing residues 722-737, 735-750, and 747-762, respectively. The bars labeled with β_3-3S, β_3-2S, β_3-1S, and β_3-4S represent adhesion of cells incubated with the cell-permeable peptides containing signal sequence hydrophobic region of integrin β_3 (Glycoprotein IIIa) followed by residues 722-737, 735-750, 747-762, and 742-755, respectively. Data are the mean ± standard error of the mean (SEM) from at least three independent experiments performed in triplicate. The differences in adhesion between control cells preincubated in the absence of peptides and cells treated with β_3-1S peptide are statistically significant at $p \leq 0.002$ (Student t test).

FIG 3. Intracellular location of cell-permeable β_3-1S peptide as demonstrated by confocal laser scanning microscopy (mid-cell 1μm section). Intracellular peptide was detected as yellow stains by indirect immunofluorescence assay and analyzed by a six-step Z-position sectional scanning of the cell. The top picture shows minimal staining of HEL cells treated with non-cell-permeable β_3-1 peptide. In the bottom, HEL cells treated with cell-permeable β_3-1S peptide clearly show a gain in fluorescent signal representing peptide in the cytoplasm of the HEL cells. Similar pattern was obtained with cells treated with cell-permeable β_3-2S and β_3-3S peptides. The anti-peptide β_3-1 antibody used for detection of cell-permeable β_3-1S peptide was monospecific (see Examples).

FIG 4. Inhibition of cell adhesion by cell-permeable peptides is integrin-specific and concentration-dependent. Analysis of the cell-permeable peptides β_3-1S and β_1-1S in a quantitative adhesion assay of HEL cells (A), ECV 304 cells (B) and HF cells (C). HEL and ECV 304 cells adhered to immobilized fibrinogen and HF cells adhered to uncoated plastic. Import of peptides and cell adhesion assay were performed as in FIG 2. Data are the mean ± SEM from at least three experiments performed in triplicate. The differences between β_3-1S peptide and β_1-1S peptide at 200μM were significant at $p < 0.0001$ for both HEL and ECV 304 cells. The difference between β_3-1S peptide and β_1-1S peptide at 200μM were significant at $p<0.0001$ for the HF cell line.

FIG 5. Effect of cell-permeable mutant peptides β_3-1S ($Ser^{752}Pro$) and β_3-1S ($Ser^{752}Ala$) on adhesion of HEL cells (○,□,△) and ECV 304 cells (●,■,▲) to immobilized fibrinogen. Import of peptides and the cell adhesion assay were performed as in FIG 2. Data are the mean ±SEM from at least three experiments performed in triplicate. The differences between β_3-1S peptide and its mutant β_3-1S ($Ser^{752}Pro$) were significant at $p < 0.0001$ for both HEL and ECV 304 cells. The difference between β_3-1S and β_3-1S ($Ser^{752}Ala$) was not significant.

FIG 6. Effect of cell-permeable mutant peptides β_3-1S ($Tyr^{747}Phe$), β_3-1S ($Tyr^{759}Phe$), and β_3-1S ($Tyr^{747/759}Phe$) on adhesion of HEL (A) and ECV 304 (B) cells to immobilized fibrinogen. Import of peptides and the cell adhesion assay were performed as in FIG 2. Data are the mean ±SEM from at least three experiments performed in triplicate. The differences between β_3-1S peptide and all three β_3-1S mutant Tyr→Phe peptides were significant at $p < 0.0001$ for both HEL and ECV 304 cells.

FIG. 7 shows a schematic presentation of the approach to the structure-function analysis of cytoplasmic tails, here specifically of integrin α_{IIb} β_3 subunits. Sequences of hydrophobic signal peptide (SEQ ID NO:1), and peptides β_3-1 (SEQ ID NO:2), β_3-2 (SEQ ID NO:3), β_3-3 (SEQ ID NO:4) and β_3-4 (SEQ ID NO:5) are shown.

FIG. 8 shows the effect of cell-permeable peptide β_3-1S in the absence (•) or the presence (▪) of verapamil in HEL cells.

FIG. 9 shows inhibition of HEL cell adhesion to fibrinogen by cell-permeable peptides β_3-1S and α_{IIb} is concentration dependent.

FIG. 10 shows the β_2 peptide and β_2-3 (residues 724-741; SEQ ID NO:9), β_2-2 (residues 7740-756; SEQ ID NO:8) and β_2-1 (SEQ ID NO:7) peptides, as well as signal sequence (SEQ ID NO:1). Additional β_2 peptides are β_2-4 (LFKSATTTVMNPKFAES (residues 753-769; SEQ ID NO:20)) and β_2-5 (KEKLKSQWNNDNPLF (residues 740-754; SEQ ID NO:21)).

DETAILED DESCRIPTION OF THE INVENTION

As used in the specification and in the claims, "a" can mean one or more, depending upon the context in which it is used.

Analysis of the 41 residue cytoplasmic tail of integrin β_3 was undertaken by applying our recently developed cell-permeable peptide import technique (10) to probe integrin β_3 cytoplasmic protein-protein interactions. As a functional endpoint, adhesion of human erythroleukemia (HEL) cells to immobilized fibrinogen in response to stimulation with 4β-phorbol 12-myristate 13-acetate (PMA) was used. HEL cells express endogenous integrin $\alpha_{IIb}\beta_3$ and serve as a useful model for structure-function studies of platelet constituents (11,12). The integrin β_3 is also expressed as a heterodimer with integrin α_v in human platelets and endothelial cells (13). Therefore, adhesion of the ECV 304 cell line derived from human umbilical vein endothelial cells that express $\alpha_v\beta_3$ integrin (vitronectin receptor) (14) was studied. Using cell-permeable peptides representing wild-type and mutated sequences, the present invention identifies the major Cell Adhesion Regulatory Domain (CARD) of integrin β_3 . It encompasses a 16 amino acid sequence of its cytoplasmic tail. A synthetic peptide mimetic representing CARD imported by HEL and ECV 304 cells inhibits "from within" their adhesion to immobilized fibrinogen by competing with intracellular protein-protein interactions involving the integrin β_3 cytoplasmic tail.

The present invention reports the structure-function analysis of the cytoplasmic tail of integrin subunits β_1, β_2 , β_3 and α_{IIb} based on the cellular import of synthetic peptide analogs of this region. Among the four overlapping cell-permeable peptides of β_3 only the peptide carrying residues 747-762 of the carboxy-terminal segment of integrin β_3 inhibited adhesion of human erythroleukemia (HEL) cells and of human endothelial cells (ECV) 304 to immobilized fibrinogen mediated by integrin β_3 heterodimers, $\alpha_{IIb}\beta_3$, and $\alpha_v\beta_3$, respectively. Inhibition of adhesion was integrin-specific because the cell-permeable β_3 peptide (residues 747-762) did not inhibit adhesion of human fibroblasts mediated by integrin β_1 heterodimers. Conversely, a cell-permeable

peptide representing homologous portion of the integrin β_1 cytoplasmic tail (residues 788-803) inhibited adhesion of human fibroblasts, whereas it was without effect on adhesion of HEL or ECV 304 cells.

The cell-permeable integrin β_3 (Glycoprotein IIIa) peptide (residues 747-762) carrying a known loss-of-function mutation ($Ser^{752}Pro$) responsible for the genetic disorder Glanzmann's thrombasthenia Paris I did not inhibit cell adhesion of HEL or ECV 304 cells, while the β_3 peptide carrying a $Ser^{752}Ala$ mutation was inhibitory. Although Ser^{752} is not essential, Tyr^{747} and Tyr^{759} form a functionally active tandem because conservative mutations $Tyr^{747}Phe$ or $Tyr^{759}Phe$ resulted in a non-functional cell permeable integrin β_3 peptide. It is herein demonstrated that the carboxy terminal segment of the integrin β_3 cytoplasmic tail spanning residues 747-762 constitutes a major intracellular Cell Adhesion Regulatory Domain (CARD) that modulates the adhesion of integrin β_3-expressing cells with immobilized fibrinogen. It is further demonstrated that the carboxy-terminal segment of the integrin β_1 cytoplasmic tail (residues 788-803) constitutes a major intracellular Cell Adhesion Regulatory Domain (CARD) that modulates the adhesion of integrin β_1-expressing cells. Additionally shown is a CARD of the integrin β_2 subunit is within a peptide comprising amino acids 724-769. Furthermore, a cytoplasmic segment containing amino acids 989-1008 of integrin subunit α_{IIb}. was tested. Import of cell-permeable peptides carrying a CARD domain results in inhibition "from within" of the adhesive function of these integrins in cells expressing these integrins. The present invention includes the basic premise that the cytoplasmic segment of the integrin β_2 subunit and its α subunit counterparts, α_L, α_M, and α_X, carry a functionally related CARD, and that the cytoplasmic segment of the integrin β_1 subunit and its α subunit counterparts, carry a functionally related CARD. A CARD for any selected adhesion receptor, adhesion molecule or counter receptor can readily be developed following the procedures herein.

The present invention provides a method of inhibiting adhesion of a cell comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by the cell.

The adhesion receptor can be an integrin. When the adhesion receptor is an integrin, the subunit can be a β subunit of an integrin. In particular, the β subunit can be β_3, β_2, or β_1, or example. The subunit can be an α subunit of an integrin. In particular it can be, for example, α_{IIb} subunit.

The adhesion receptor can be a selectin. The adhesion receptor can be a cell adhesion molecule such as an ICAM, *e.g.*, ICAM-1. ICAM-2, ICAM-3.

The present invention provides a method of inhibiting or disrupting cellular adhesion of a cell comprising transferring into the cell a polypeptide comprising a cell adhesion regulatory domain of an adhesion receptor or counter receptor expressed by the cell. In particular, the present invention provides a method of inhibiting or disrupting cellular adhesion of a cell comprising transferring into the cell a polypeptide comprising a cell adhesion regulatory domain of a subunit, *i.e.*, the α subunit or the β subunit of an integrin expressed by the cell.

In particular, the present invention provides a method of reducing or preventing excessive proliferation of a fibroblast comprising transferring into the fibroblast a peptide comprising a cell adhesion regulatory domain of a β_1 subunit of an integrin receptor. The administration of the peptide disrupts adhesion, thus reducing or preventing adhesion-dependent proliferation of the cells. For example, a peptide comprising the amino acid sequence set forth in SEQ ID NO:6 (peptide β_1-1) can be used in this method. Such inhibition of excessive fibroblast proliferation can be used to promote wound healing.

Because adhesion receptors and counter receptor mediate various forms of cellular adhesion, the present method can be utilized to disrupt these various forms of adhesion. Therefore, for example, the present method can be used to disrupt cell-substratum adhesion, cell-cell aggregation, and/or direct cell-cell adhesion. The present method can be used to transfer the peptide into a cell *in vitro*; *ex vivo*, to ultimately transfer the cell into a subject; and *in vivo* directly into a cell in a subject.

Several integrins and the cells expressing particular integrins are known in the art (*see*,, *e.g.*, Hynes, R.O. 1992 *Cell 69*:11-25; Felding-Habermann, B. and D.A. Cheresh 1993 *Curr. Opin. Cell Biol. 5*:863-868; Albeda and Buck 1990 *FASEB J. 4*:2868-2880; Arnaout 1990 *Blood 75*:1037-1050; Hemler 1990 *Annu. Rev. Immunol.8*:365-400; Springer 1990 *Nature 346*:425-434; Ruoslahti 1991 *J. Clin Invest. 87*:1-5). An integrin is an αβ heterodimer. The α subunits vary in size (between about 120 and 180 kD) and are each covalently associated with a β subunit. Currently about 8 β subunits are characterized and about 14 α subunits are characterized. Thus, integrins can include an α chain selected from, for example, vertebrate α_V, α_1, α_2, α_3, α_4,α_5, α_6, α_7, α_8, α_L, α_M, α_{IIb}, α_{IEL}, and α_X, and a β chain from, for example, β_1, β_2, β_3, β_4, β_6, β_7, and β_8. Thus, for example, a cell adhesion regulatory domain of a β_1 subunit can be used to disrupt cellular adhesion of a cell expressing, for example, $\alpha_1\beta_1$ to $\alpha_8\beta_1$ or $\alpha_V\beta_1$ integrin. Thus, for example, a cell adhesion regulatory domain of a β_2 subunit can be used to disrupt cellular adhesion of a cell expressing, for example, $\alpha_L\beta_2$, $\alpha_M\beta_2$, or $\alpha_X\beta_2$ integrin. Also, for example, a cell adhesion regulatory domain of a β_3 subunit can be used to disrupt cellular adhesion of a cell expressing, for example, $\alpha_{IIb}\beta_3$ or $\alpha_V\beta_3$ integrin. Additionally, for example, a cell adhesion regulatory domain of a β_1 subunit can be used to disrupt cellular adhesion of a cell expressing, for example, $\alpha_5\beta_1$ or $\alpha_2\beta_1$. Furthermore, a cell adhesion regulatory domain of an α_{IIb} subunit can be used to inhibit, for example, $\alpha_{IIb}\beta_3$ integrin. The present method can disrupt cellular adhesion of an integrin regardless of the extracellular binding region of the receptor and regardless of the recognition sequence on the target protein recognized by the integrin receptor because the present method disrupts binding by disrupting the intracellular domain of the receptor. Therefore, the binding sites for the receptors need not be fully characterized.

Most integrins are expressed on a wide variety of cells. Most cells express several integrins. The present invention can be utilized for any cell type as long as the polypeptide comprising the appropriate cell adhesion regulatory domain can be transferred into the cell and the cell expresses the integrin. Mechanisms particularly effective for transferring such proteins (by means of transferring the protein or the nucleic acid encoding it) are known in the art for specific cell types, and additionally

some are exemplified herein. (*see, e.g.*, Hynes, R.O. 1992 Cell 69:11-25; Felding-Habermann, B. and D.A. Cheresh 1993 *Curr. Opin. Cell Biol. 5*:863-868; Albeda and Buck 1990 *FASEB J. 4*:2868-2880)

Selectins, cell surface adhesion molecules, for example, L-selectin, (Tedder TF, et al.J. Experimental Medicine 1995 June;181:2259-2264), can also be inhibited by this method to disrupt cellular adhesion. The CARD of any given selectin can be readily determined following the methods taught herein as exemplified by determining the CARDs of the β_1 and β_3 subunits of integrins. Briefly, one can introduce cytoplasmic segments into appropriate cells expressing the receptor or counter receptor and test for inhibition of adhesion as demonstrated in the examples. Using selectin CARD peptides, one can target adhesion disruption specifically in cells expressing the particular selectin. For example, L-selectin is expressed by most leukocytes, E-selectins are expressed by endothelial cells, and P-selectins are expressed by platelets.

Cadherins, calcium-dependent adhesion receptors, can also be inhibited by this method to disrupt cellular adhesion. The CARD of any given cadherin, such as E-cadherin, N-cadherin and P-cadherin, can be readily determined following the methods taught herein as exemplified by determining the CARDs of the β_1 and β_3 subunits of integrins. Briefly, one can introduce cytoplasmic segments into appropriate cells expressing the receptor or counter receptor and test for inhibition of adhesion as demonstrated in the examples. Using cadherin CARD peptides, one can target adhesion disruption specifically in cells expressing the particular selectin. For example, E-cadherins are expressed by endothelial cells, N-cadherins are expressed on neuronal cells and P-cadherins are expressed by platelets.

Cell adhesion molecules such as ICAMs can also be used in the present method. ICAM CARDs can readily be determined following the methods presented herein. Using ICAM CARD peptides, one can target adhesion disruption specifically in cells expressing the particular ICAM, for example, epithelial cells, endothelial cells, or leukocytes.

Transfer of the polypeptide into the cell can be accomplished by any selected means. For example, a signal peptide can be linked to a polypeptide comprising the cell adhesion regulatory domain and this complex administered to the cell. The signal peptide can be linked, e.g., by constructing a recombinant vector encoding a chimeric peptide comprising the signal peptide and the polypeptide comprising the cell adhesion regulatory domain. This recombinant vector can then be expressed *ex vivo*, after which the protein would be transferred into the cell to be disrupted, aided in cell entry by the signal peptide. This mode of transfer is exemplified in the examples herein. The signal peptide can also be linked to the polypeptide by other means such as a chemical link (see, e.g., U.S. Serial No. 08/258,852).

Furthermore, other hydrophobic sequences other than signal peptides, can be used to deliver the peptide into the cell. Helical structures in homeobox proteins can be used. Any such peptide for transfer of the peptide into a cell can be generated as part of a chimeric peptide; it can be linked by a peptide (amide) bond; it can be cross-linked chemically.

Additionally, a nucleic acid encoding the peptide comprising the cell adhesion regulatory domain can be constructed such that the coding region is functionally linked to a promoter compatible with the cell in which adhesion is to be disrupted, and the vector then transferred into the cell. The cell can then produce the peptide itself. If such *in vivo* expression is utilized, the vector can be selected such that the vector can be readily transferred into the specific cell type. For example, an adenoviral vector, an adeno-associated viral vector or a retroviral could be utilized to transfect any of several types of cells. Other viral vectors have cell specificity as known in the art, and can be utilized accordingly for targeting such cells.

A suitable nucleotide sequence for a nucleic acid encoding a CARD peptide can readily be deduced from the amino acid sequence of the CARD, as is standard in the art. Additionally, species-preferred codons can be utilized for the particular species whose

cell is to be transferred for optimal expression of the peptide. Such species codon specificities are known in the art.

Alternatively other transfection means can be utilized separately or in conjunction with that exemplified herein to achieve global or selective transfer of the vector. For example, a cationic liposome composition comprising the CARD peptide alone, the chimeric signal peptide-CARD peptide, or a nucleic acid encoding the CARD peptide can be utilized particularly to target the lungs, upon inhalation or intravenous injection (Brigham, *et al. Amer. J. Respir. Cell and Mol. Biol.* 8:209-213 (1993); Felgner *et al.*, 1987 *Proc. Natl. Acad. Sci. U.S.A.* **84**:7413; U.S. Patent No. 4,897,355 (Eppstein, et al.)) . By way of another example, liposome compositions can be utilized that have incorporated proteins that have specific receptors on the target cells. Additionally, the promoter of the vector can also be selected such that expression from it can be induced or expressed in only particular cell types. Inducible promoters include, for example, the metallothionein promoter, which can be induced by exposure to zinc, such as in the diet of the subject. Many such selectively or inducibly expressed promoters are known in the art.

Cationic and anionic liposomes are contemplated for use in this invention, as well as liposomes having neutral lipids. Cationic liposomes can be complexed with the CARD peptide, the signal peptide-CARD peptide chimeric peptide, or the nucleic acid encoding the CARD peptide by mixing these components and allowing them to charge-associate. Cationic liposomes are particularly useful with a nucleic acid because of the nucleic acid's negative charge. Examples of cationic liposomes include lipofectin, lipofectamine, lipofectace and DOTAP.[32a-34a] Anionic liposomes generally are utilized to encase within the liposome the substances to be delivered to the cell. Procedures for forming cationic liposomes encasing substances are standard in the art[35a] and can readily be utilized herein by one of ordinary skill in the art to encase the complex of this invention.

For peptide import using an importation-competent signal peptide, suitable import conditions are exemplified herein and include cell and complex temperature between about 18°C and about 42°C, with a preferred temperature being between about 22°C and about 37°C. For administration to a cell in a subject, the complex, once in the subject, will of course adjust to the subject's body temperature. For *ex vivo* administration, the complex can be administered by any standard methods that would maintain viability of the cells, such as by adding it to culture medium (appropriate for the target cells) and adding this medium directly to the cells. As is known in the art, any medium used in this method can be aqueous and non-toxic so as not to render the cells non-viable. In addition, it can contain standard nutrients for maintaining viability of cells, if desired. For *in vivo* administration, the complex can be added to, for example, a blood sample or a tissue sample from the patient, or to a pharmaceutically acceptable carrier, e.g., saline and buffered saline, and administered by any of several means known in the art. Examples of administration include parenteral administration, e.g., by intravenous injection including regional perfusion through a blood vessel supplying the tissues(s) or organ(s) having the target cell(s), or by inhalation of an aerosol, subcutaneous or intramuscular injection, topical administration such as to skin wounds and lesions, direct transfection into, e.g., bone marrow cells prepared for transplantation and subsequent transplantation into the subject, and direct transfection into an organ that is subsequently transplanted into the subject. Further administration methods include oral administration, particularly when the composition is encapsulated, or rectal administration, particularly when the composition is in suppository form. A pharmaceutically acceptable carrier includes any material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the selected complex without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

Administration can be performed for a time length of about 1 minute to about 72 hours. Preferable time lengths are about 5 minutes to about 48 hours, and even more preferably about 5 minutes to about 20 hours, and even more preferably

about 5 minutes to about 2 hours. Optimal time lengths and conditions for any specific complex and any specific target cell can readily be determined, given the teachings herein and knowledge in the art.[27a] Specifically, if a particular cell type *in vivo* is to be targeted, for example, by regional perfusion of an organ or tumor, cells from the target tissue can be biopsied and optimal dosages for import of the complex into that tissue can be determined *in vitro*, as described herein and as known in the art, to optimize the *in vivo* dosage, including concentration and time length. Alternatively, culture cells of the same cell type can also be used to optimize the dosage for the target cells *in vivo*.

For either *ex vivo* or *in vivo* use, the complex can be administered at any effective concentration. An effective concentration is that amount that results in importation of the biologically active molecule into the cell. Such a concentration will typically be between about 0.5 nM to about 100 μM (culture medium concentration (*ex vivo*) or blood serum concentration (*in vivo*)). Optimal concentrations for a particular complex and/or a particular target cell can be readily determined following the teachings herein. Thus, *in vivo* dosages of the complex include those which will cause the blood serum concentration of the complex to be about 0.5 nM to about 100 μM. A preferable concentration is about 2 nM to about 50 μM. The amount of the complex administered will, of course, depend upon the subject being treated, the subject's age and weight, the manner of administration, and the judgment of the skilled administrator. The exact amount of the complex will further depend upon the general condition of the subject, the severity of the disease/condition being treated by the administration and the particular complex chosen. However, an appropriate amount can be determined by one of ordinary skill in the art using routine optimization given the teachings herein.

Parenteral administration, e.g., regional perfusion, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, such as liquid solutions, suspensions, or emulsions. A slow release or sustained release system, such as disclosed in U.S. Patent No. 3,710,795, can also be used, allowing the maintenance of a constant level of dosage.

Depending on the intended mode of administration, the pharmaceutical compositions may be in the form of solid, semi-solid or liquid dosage forms, such as, for example, tablets, suppositories, pills, capsules, powders, liquids, suspensions, lotions, creams, gels, or the like, preferably in unit dosage form suitable for single administration of a precise dosage. The compositions will include, as noted above, an effective amount of the selected drug in combination with a pharmaceutically acceptable carrier and, in addition, may include other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc.

For solid compositions, conventional nontoxic solid carriers include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talc, cellulose, glucose, sucrose, magnesium carbonate, and the like. Liquid pharmaceutically administrable compositions can, for example, be prepared by dissolving, dispersing, etc. an active compound as described herein and optional pharmaceutical adjuvants in an excipient, such as, for example, water, saline, aqueous dextrose, glycerol, ethanol, and the like, to thereby form a solution or suspension. If desired, the pharmaceutical composition to be administered may also contain minor amounts of nontoxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see *Remington's Pharmaceutical Sciences*.[27a]

An "importation competent signal peptide," as used herein, is a sequence of amino acids generally of a length of about 10 to about 50 or more amino acid residues, many (typically about 55-60%) residues of which are hydrophobic such that they have a hydrophobic, lipid-soluble portion.[1a] The hydrophobic portion is a common, major motif of the signal peptide, and it is often a central part of the signal peptide of protein secreted from cells. A signal peptide is a peptide capable of penetrating through the cell membrane to allow the export of cellular proteins. The signal peptides of this invention, as discovered herein, are also "importation competent," i.e., capable of penetrating through the cell membrane from outside the cell to the interior of the cell. The amino

acid residues can be mutated and/or modified (i.e., to form mimetics) so long as the modifications do not affect the translocation-mediating function of the peptide. Thus the word "peptide" includes mimetics and the word "amino acid" includes modified amino acids, as used herein, unusual amino acids, and D-form amino acids. All importation competent signal peptides encompassed by this invention have the function of mediating translocation across a cell membrane from outside the cell to the interior of the cell. Such importation competent signal peptides could potentially be modified such that they lose the ability to export a protein but maintain the ability to import molecules into the cell. A putative signal peptide can easily be tested for this importation activity following the teachings provided herein, including testing for specificity for any selected cell type.

Signal peptides can be selected, for example, from the SIGPEP database, which also lists the origin of the signal peptide.[30a, 38a] When a specific cell type is to be targeted, a signal peptide used by that cell type can be chosen. For example, signal peptides encoded by a particular oncogene can be selected for use in targeting cells in which the oncogene is expressed. Additionally, signal peptides endogenous to the cell type can be chosen for importing biologically active molecules into that cell type. And again, any selected signal peptide can be routinely tested for the ability to translocate across the cell membrane of any given cell type according to the teachings herein. Specifically, the signal peptide of choice can be conjugated to a peptide, *e.g.*, a CARD of a cellular adhesion receptor, adhesion molecule or counter receptor, and administered to a cell, and the cell is subsequently screened for the presence of the CARD peptide.

The presence of modified amino acids in the signal peptide can additionally be useful for rendering a peptide, polypeptide or protein more resistant to peptidases in the subject. Thus these signal peptides can allow for more effective treatment by allowing more peptides to reach their target and by prolonging the life of the peptide before it is degraded. Additionally, one can modify the amino acid sequence of the signal peptide to alter any proteolytic cleavage site present in the original signal sequence for removing the signal sequence. Cleavage sites are characterized by small,

positively charged amino acids with no side chains and are localized within about 1 to about 4 amino acids from the carboxy end of the signal peptide.[1a]

An example of a useful signal peptide is the signal peptide from human integrin β_3: VTVLALGALAGVGVG (SEQ ID NO:1) (hydrophobic region (h-region) of the signal peptide sequence of human integrin subunit β_3) (6,7). Another example is the signal peptide from Kaposi fibroblast growth factor (K-FGF)[16a-17a] : Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro (SEQ ID NO:17). Any signal peptide, however, capable of translocating across the cell membrane into the interior of the selected target cell can be used according to this invention.

The cell into which the CARD peptide is transferred can be any cell expressing the adhesion molecule or counter receptor. For example, the cell can be a white blood cell; a granulocyte, a monocyte; a lymphocyte; it can also be a cancer cell, specifically a tumor cell, a leukemia cell. Cancer cell includes cells of a tumor mass and metastatic cells. Also for β_3: platelets, endothelial cells; for β_1: fibroblasts. Cells can be within a tissue or organ, for example, supplied by a blood vessel into which the complex is administered. Additionally, the cell can be targeted by, for example, inhalation of the molecule linked to the peptide to target the lung epithelium, or ingestion or suppository administration to target the intestinal epithelium. In addition, the chimeric peptide or nucleic acid encoding the CARD peptide can be administered directly to a tissue site in the body. As discussed above, the signal peptide utilized can be chosen from signal peptides known to be utilized by the selected target cell, or a desired signal peptide can be tested for importing ability given the teachings herein. Generally, however, all signal peptides have the common ability to cross cell membranes due, at least in part, to their hydrophobic character. Thus, in general, a membrane-permeable signal peptide can be designed and used for any cell type, since all eukaryotic cell membranes have a similar lipid bilayer.

The effective concentration of imported peptide or its mimetic is an amount sufficient to inhibit cellular adhesion. Experimentally, one can calculate the

concentration of CARD peptide necessary *in vitro* to cause 50% inhibition of, *e.g.*, fibroblast or endothelial cells.

Additionally, the concentration needed to achieve sufficient transfer can be reduced by employing methods to increase intracellular concentration of peptides. For example, as shown herein, cells can be treated with a compound that prevents active transport of peptides from the cell. Herein it is shown that blockers of the MDR pump, such as chemotherapeutic drug analogs, calcium channel antagonists (*e.g.*, verapamil), immunosuppressive cyclic peptides (*e.g.*, cyclosporin), and calmodulin inhibitors also block the removal of peptides from the cell. Thus, in combination with the treatment of cells with blockers of MDR pump or similar membrane proteins that remove drugs, peptides, etc. from cells, the amount of CARD peptide to be administered can be reduced. Thus, effectiveness of imported peptides can be increased while any potential side effects can be minimized. Often a subject may already be taking an MDR pump blocker; thus this combination treatment can have minimal adverse effect. The amount of blocker can be readily determined using standard methods, in particular as exemplified herein. A typical dosage will be that amount used in the art for verapamil or cyclosporin administration.

The cell adhesion regulatory domain of an adhesion receptor or adhesion molecule, such as an integrin, a cadherin or a selectin, or of a counter receptor is a peptide, or mimetic thereof, comprising the cytoplasmic portion of a subunit of the adhesion receptor, adhesion molecule or counter receptor which, when transferred into a cell expressing the adhesion receptor, adhesion molecule or counter receptor from which the peptide was derived, inhibits binding of the adhesion receptor or adhesion molecule to its ligand or counter receptor or of the counter receptor to its corresponding receptor. This portion is herein referred to as the cytoplasmic adhesion regulatory domain (CARD). For example, for an integrin, which has an α subunit and a β subunit, a composition comprising a peptide comprising a CARD of the α subunit can be used or a composition comprising a peptide comprising a CARD of the β subunit can be used. For example, for a receptor having the integrin β_3 chain, a peptide comprising amino

acids 747-762 of the integrin β_3 chain can be used: YKEATSTFTNITYRGT (SEQ ID NO:2- residues 747-762 of integrin subunit β_3 (peptide β_3-1)) or VTVLALGALAGVGVGYKEATSTFTNITYRGT (SEQ ID NO:11-signal sequence linked to residues 747-762 of integrin subunit β_3 at N-terminus (peptide β_3-1S)); for a receptor having the integrin β_1 chain, a peptide comprising amino acids 788-803 of the integrin β_1 chain can be used: YKSAVTTVVNPKYEGK (SEQ ID NO:6- residues 788-803 of integrin subunit β_1 (peptide β_1-1)) or VTVLALGALAGVGVGYKSAVTTVVNPKYEGK (SEQ ID NO:12- signal sequence linked to residues 788-803 of integrin subunit β_1 at N-terminus (peptide β_1-1S)); for a receptor having the integrin β_2 chain, a peptide comprising amino acids 724-769 of the integrin β_2 chain can be used: KALIHLSDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES (SEQ ID NO:18- residues 724-769 of integrin subunit β_2). A shorter β_2 peptide retaining cell adhesion inhibiting activity can be readily derived following the examples herein. For example, peptides β_2-1 (SEQ ID NO:7), β_2-2 (SEQ ID NO:8), β_2-3 (SEQ ID NO:9), β_2-4 (SEQ ID NO:20), or β_2-5 (SEQ ID NO:21) can be used in the provided methods to further delineate the integrin β_2 CARD. For a receptor having the integrin α_{IIb} subunit, a peptide comprising amino acids 989-1008 can be used: KVGFFKRNRPPLEEDDEEGE (SEQ ID NO:10- peptide α_{IIb} (residues 989-1008 of integrin subunit α_{IIb})) or VTVLALGALAGVGVGKVGFFKRNRPPLEEDDEEGE (SEQ ID NO:14- signal sequence linked to residues 989-1008 of integrin subunit α_{IIb} at N-terminus (peptide α_{IIb} S)). For integrins α_L, α_M, and α_X and ICAM-1, ICAM-2, ICAM-3, and L-selectin, E-selectin, and P-selectin, or any other adhesion receptor or counter receptor, cytoplasmic segments of the subunit can be used as adhesion inhibitory peptides when imported into cells expressing the receptor or counter receptor. Additionally, shorter peptides can be tested for such activity and used.

A peptide comprising the cell adhesion regulatory domain can have preferably conservative amino acid substitutions and or deletions as long as the domain retains its ability to inhibit binding of the receptor to its ligand and/or counter receptor. Useful substitutions and deletions can be readily determined by following the teaching of the

examples. For example, as found by the methods in the Examples, a polypeptide including amino acids 747-762 of the integrin β_3 chain can include a Serine to Alanine substitution at amino acid 752; however, a Serine to Proline substitution at amino acid 752, a less conservative substitution, results in a peptide that is inactive in the function of inhibiting binding of the receptor to its ligand. By following this method, for which an example of an acceptable substitution and an unacceptable substitution is provided, other acceptable substitutions can be obtained and thus such substituted peptides are within the scope of this invention. Additionally, as known in the art, conservative and non-conservative substitutions can be predicted.

A mimetic of a CARD peptide can be designed based upon the conformational characteristics of the CARD peptide. For example, the CARD peptide can be crystallized and the characteristics of the peptide determined using a computer program designed for such functions. A computer program can then be run to predict substitutions in the molecule that can be made for specific amino acids in the peptide to retain the binding conformation. The resulting mimetic can be a peptide mimetic or it can have other molecules substituted in place of at least one or more of the amino acids. Mimetics can be designed, for example, to be more resistant to degradation in the body.

The present invention further provides a method of reducing or preventing an inflammatory response of a cell comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor, adhesion molecule or counter receptor expressed by the cell. The adhesion receptor, adhesion molecule or counter receptor can be any adhesion receptor, adhesion molecule or counter receptor expressed by the target cell of interest. For example, the adhesion receptor can be an integrin. The subunit can be a β subunit of an integrin expressed by the cell. The subunit can be an α subunit of an integrin expressed by the cell.

The present invention further provides a method of reducing or preventing blood clotting in a subject comprising contacting a platelet with a chimeric peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory

domain (CARD) of a β subunit of an integrin, thereby reducing or preventing platelet cell adhesion and reducing or preventing blood clotting in the subject. In particular, the CARD can be of a β1, β2, or a β3 subunit of an integrin.

The present invention further provides a method of reducing or preventing blood clotting in a subject comprising contacting *ex vivo* a platelet from the subject with a chimeric peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, and replacing the platelet into the subject, thereby reducing or preventing platelet cell adhesion and reducing or preventing blood clotting in the subject.

The instant invention additionally provides a method of reducing or preventing platelet cell adhesion in a subject comprising contacting a platelet with a chimeric peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, thereby reducing or preventing platelet cell adhesion in the subject.

The peptide can be administered *in vivo* or *ex vivo* to cells removed from the subject and then injected back into the subject once the peptide is transferred. For example, if a patient is undergoing a procedure such as pheresis or dialysis, the peptide can be administered to the blood as it is removed. If a subject is undergoing a procedure such as a removal of a tumor, the peptide can be directly infused to the site of tumor removal, to inhibit regrowth of the tumor and to inhibit metastasis. Furthermore, if a subject is undergoing open heart surgery or angioplasty, the peptide can be administered directly at the site of injury to the heart and/or blood vessel, to prevent inflammation or restenosis. A subject with adult respiratory distress syndrome can be administered by inhalation of the peptide in a liposome composition in a manner and in dosages similar to that used for applying CFTR to the lungs in cystic fibrosis patients. Also, for adult respiratory distress syndrome, the peptide can be administered intravenously. Furthermore, for intestinal applications, the peptide can be placed in a carrier resistant to

pepsin and taken orally; alternatively the peptide can be administered such that it proceeds directly to the intestine with minimal dwell time in the stomach.

If a patient is undergoing a procedure involving extracorporeal circulation of the patient's blood, the present method can be used to prevent platelet adhesion in the subject's blood while it is circulating in the equipment for the extracorporeal circulation. Therefore, the present invention provides a method of reducing or preventing platelet adhesion in extracorporeal circulation of a subject comprising contacting *ex vivo* during the extracorporeal circulation process a platelet from the subject with a chimeric peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, and replacing the platelet into the subject, thereby reducing or preventing platelet cell adhesion in the extracorporeal circulation.

The present invention provides a method of treating or preventing coronary and/or vascular disease or conditions in a subject comprising administering to the subject a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by vascular endothelial cells, thereby reducing or inhibiting attachment of leukocytes and platelets to an atherosclerotic lesion.

It is preferred that the peptide be administered prior to formation of the lesion. For example, it can be administered at the time of surgery, such as angioplasty or open heart surgery, is performed on a blood vessel or on the heart.

The present invention provides a method of reducing or preventing granulocyte adhesion in a subject comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by the granulocyte, thereby reducing or preventing adhesion of the granulocyte in the subject.

This method can be performed *in vivo* or *ex vivo*. One can, for example, to transfer the peptide into the cell, contact a granulocyte of the subject with a chimeric peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, thereby reducing or preventing granulocyte adhesion in the subject.

Administration is preferably performed at the time of a surgical procedure associated with granulocyte adhesion occurring after the procedure, such as open heart surgery or angioplasty. Granulocytes and platelets adhere to damaged endothelial cells lining the blood vessels, especially in the microvasculature of the lungs; therefore, this treatment can be used to selectively affect areas having damaged endothelial cells.

The present invention provides a method of preventing or reducing atherosclerotic plaques in the blood vessels of a subject, comprising administering to the subject comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor, adhesion molecule or counter receptor expressed by an endothelial cell of the blood vessel, a granulocyte or a platelet, thereby reducing or preventing adhesion of the endothelial cells, granulocyte, and/or platelet in the subject. Such a method can be performed *in vivo* or *ex vivo* to cells removed from the subject.

The present invention provides a method of treating adult respiratory distress syndrome in a subject comprising administering to the subject a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by a granulocyte of the subject, thereby preventing or reducing adhesion of a granulocyte to the endothelial lining of the microvasculature of the lungs and treating adult respiratory distress syndrome.

Granulocyte binding to endothelial cells in blood vessels of the lungs inhibits gas exchange in the capillaries to cause adult respiratory distress syndrome and can further complicate with peptic shock. Thus, by preventing or reducing the binding of

granulocytes to the blood vessels, the respiratory distress response can be inhibited or reduced.

In any of the methods wherein a CARD peptide is transferred into a cell, the peptide can be transferred by any of several means. For example, it can be prepared in a chimeric peptide which includes at its amino-terminal end an importation-competent signal peptide. It can also be linked to an importation competent signal peptide by other means and administered as a complex. Furthermore, other standard means of administering peptides to cells can be utilized.

By "linked" as used herein is meant that the CARD peptide is associated with the signal peptide in such a manner that when the signal peptide crosses the cell membrane, the is also imported across the cell membrane. Examples of such means of linking include (1) the signal peptide can be linked by a peptide bond, i.e., the two peptides can be synthesized contiguously; (2) the signal peptide can be linked to the CARD peptide by a peptide bond or by a non-peptide covalent bond (such as conjugating a signal peptide to a CARD peptide with a crosslinking reagent); (3) the CARD peptide and the signal peptide can be joined by charge-association between the negatively-charged amino acids in the CARD peptide and the positively-charged amino acids in the peptide; (4) chemical ligation methods can be employed to create a covalent bond between the carboxy-terminal amino acid of the signal peptide and the CARD peptide. Methods (1) and (2) are typically preferred.

Examples of method (1) are shown below wherein a peptide is synthesized, by standard means known in the art,[24a,25a] that contains, in linear order from the amino-terminal end, a signal peptide sequence and a CARD peptide. Such a peptide could also be produced through recombinant DNA techniques, expressed from a recombinant construct encoding the above-described amino acids to create the peptide.[28a]

For method (2), either a peptide bond, as above, can be utilized or a non-peptide covalent bond can be used to link the signal peptide with the CARD peptide. This non-peptide covalent bond can be formed by methods standard in the art, such as by conjugating the signal peptide to the CARD peptide via a crosslinking reagent, for example, glutaraldehyde. Such methods are standard in the art.[29a] For method (3) the peptide can simply be mixed with the signal peptide and thus allowed to associate. These methods are performed in the same manner as association of peptides with cationic liposomes.[32a-34a] Such methods are standard in the art.

For method (4), standard chemical ligation methods, such as using chemical crosslinkers interacting with the carboxy-terminal amino acid of the signal peptide, can be utilized. Such methods are standard in the art (*see*, e.g., Goodfriend,[31a] which uses water-soluble carbodiimide as a ligating reagent) and can readily be performed to link the carboxy terminal end of the signal peptide to any selected CARD peptide.

The present invention provides a peptide comprising a cell adhesion regulatory domain of a β subunit of an integrin. The β subunit can be β_1. The β subunit can comprise amino acids 788-803 of β_1 subunit (SEQ ID NO:6). The β subunit can be β_3. The β subunit can comprise amino acids 747-762 of β_3 subunit (SEQ ID NO:2). The β subunit can be β_2. The β subunit can comprise amino acids 724-769 of β_2 subunit (SEQ ID NO:18). The present invention also provides a peptide comprising a cell adhesion regulatory domain of an α subunit of an integrin. The α subunit can be α_{IIb} subunit. The α subunit can comprise amino acids 989-1008 of α_{IIb} subunit (SEQ ID NO:10).

The invention further provides a chimeric peptide comprising an importation-competent peptide linked at the N-terminus to a cell adhesion regulatory domain of an adhesion receptor, adhesion molecule or counter receptor. An importation-competent peptide can be selected from any sequence that facilitates transport of proteins across a cellular membrane, such as signal peptides, hydrophobic regions of peptides, and helical structures in homeobox proteins. The invention specifically provides a chimeric peptide

comprising a signal peptide linked at the N-terminus to a cell adhesion regulatory domain of a β subunit of an integrin. The signal peptide can be the hydrophobic region (h-region) of the signal peptide sequence of human integrin β_3 : (SEQ ID NO:1), for example, or any other signal peptide or other hydrophobic sequences, as known in the art. The β subunit can be β_1. The peptide can comprise the signal peptide sequence of human integrin β_3 linked at the N-terminus to amino acids 788-803 of β_1 subunit (SEQ ID NO:12). The β subunit can be β_3. The peptide can comprise the signal peptide sequence of human integrin β_3 linked at the N-terminus to amino acids 747-762 of β_3 subunit (SEQ ID NO:11). The subunit β_3 can have a Ser^{752} to Ala^{752} substitution : YKEATATFTNITYRGT (SEQ ID NO:15). A chimeric peptide comprising the subunit β_3 can have a Ser^{752} to Ala^{752} substitution: VTVLALGALAGVGVGYKEATATFTNITYRGT (SEQ ID NO:16). The β subunit can be β_2. The chimeric peptide can comprise the signal peptide sequence of human integrin β_3 linked at the N-terminus to amino acids 724-769 of β_2 subunit (SEQ ID NO:18). Other substitutions can be determined following the methods set forth herein to determine substituted peptides that can be used to disrupt cellular adhesion.

The invention also specifically provides a chimeric peptide comprising a signal peptide linked at the N-terminus to a cell adhesion regulatory domain of an α subunit of an integrin. For example the α subunit can be α_{IIb}. The cell adhesion regulatory domain of an α subunit of an integrin can be linked to any importation-competent peptide, as exemplified herein by a signal peptide from integrin subunit β_3. The cell adhesion regulatory domain of an α_{IIb} subunit of an integrin can be the amino acid set forth in SEQ ID NO:10. A chimeric peptide can comprise the signal peptide sequence of human integrin β_3 linked at the N-terminus to amino acids 989-1008 of α_{IIb} subunit (SEQ ID NO:10), such as the chimeric peptide set forth in SEQ ID NO: 14.

The peptides of this invention can be made by any of several standard methods, such as chemical synthesis or by constructing recombinant molecules encoding the

chimeric protein, expressing and isolating the expressed chimeric protein, in a cell or in a cell-free system.

Statement concerning utility

The present invention can be used in any method and for any treatment in which inhibition of cellular adhesion can be beneficial. For example, the method can be used to prevent adhesion of leukemia cells. The method can be utilized to prevent metastatic growth of any tumors, since adhesion is required to form tumor masses. Since cancerous cells in the blood adhere to the walls of lymph nodes at very early stages of metastasis, this method can be particularly effective in cancer treatment, prevention and reduction. Additionally, the present method can be utilized to reduce or prevent inflammatory response by preventing adhesion of such cells. Furthermore, the present method can be directed to inhibiting adhesion of platelets to reduce or prevent blood clotting, and thus reduce or prevent conditions such as the progression of thrombosis and atherosclerosis. The present method can also be used to prevent adhesion of granulocytes, of endothelial cells, etc., and thus can be used to treat heart conditions, atherosclerosis, etc.

The present invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

EXAMPLES

Synthetic Peptides, Antibodies, and Cell Lines

Peptides were synthesized by a step-wise, solid-phase peptide synthesis method and purified by C_{18} reverse phase high performance liquid chromatography (HPLC) (10). As depicted in Figure 1, overlapping peptides encompassing the entire integrin β_3 cytoplasmic sequence (SEQ ID NO: 19) (6,7) represent residues 722-737 (peptide β_3-3), 735-750 (peptide β_3-2) (SEQ ID NO:4), 747-762 (peptide β_3-1) (SEQ ID NO:2), and 742-755 (peptide β_3-4) (SEQ ID NO:5). The cell-permeable peptides were

designed (10) by using the hydrophobic region Val-Thr-Val-Leu-Ala-Leu-Gly-Ala-Leu-Ala-Gly-Val-Gly-Val-Gly (h-region) (SEQ ID NO:1) of the signal peptide sequence of human integrin β_3 (6, 7) followed by the sequences of the cytoplasmic segments listed above. We also synthesized a cell-permeable peptide representing residues 788-803 of integrin β_1 (SEQ ID NO:6; Figure 1) (15,16). The molecular weights of the purified peptides were verified by mass spectrometry analysis and their composition and concentration by amino acid analysis. Polyclonal antibodies against the integrins β_3 and β_1 peptides without the hydrophobic region sequence were raised in rabbits immunized with a given peptide conjugated to keyhole limpet hemocyanin. The antibodies were monospecific for the respective β_3 peptides, as measured by ELISA. In addition, anti-integrin β_1 peptide antibody did not react with integrin β_3 peptides or with integrin $\alpha_{IIb}\beta_3$ heterodimer. Polyclonal anti-human integrin $\alpha_{IIb}\beta_3$ (Glycoprotein IIb-IIIa) antibodies were raised in rabbits using purified Glycoprotein IIb-IIIa (17). Anti-integrin β_1 monoclonal antibody (clone P4 C10) was obtained commercially from GIBCO BRL. The HEL cell line (11, 12) was obtained from Dr.Thalia Papayannopoulou, University of Washington, Seattle, Washington. Human endothelial cell line ECV304 (14) was obtained from Dr. Tom Maciag, American Red Cross Holland Laboratories, Rockville, Maryland, and human foreskin fibroblast cell line (18) was provided by Dr. Graham Carpenter, Vanderbilt University, Nashville, Tennessee.

Cell Adhesion Assay to Measure the Functional Effect of Cell-Permeable Peptides

Microtiter plates (96-well, Immulon-2, Dynatech) were coated with purified human fibrinogen (19) at 1.25 micrograms (μg)/milliliter (ml) and kept overnight at 4°C, washed with phosphate buffered saline (PBS), and incubated for 60 minutes at 37°C with 1% bovine serum albumin (BSA) to block non-specific sites. To measure the effect of the peptides on cell adhesion, HEL, ECV 304 cells or human fibroblasts (10^5 cells/well) were incubated with the indicated concentration of peptide at room temperature for 30 minutes in RPMI/10% serum, and centrifuged at 180 g. The peptide-containing supernatant was removed and cells were resuspended in RPMI/10% serum. PMA (10 nM) was added to only HEL cells, and cells were plated on fibrinogen-coated microtiter plates. Adhesion of human fibroblasts (HF)

was studied on fibrinogen-free plates. After incubation at 37°C for 120 minutes (HEL cells) and 240 minutes (ECV 304 and HF cell lines), the plates were washed 3 times with PBS and adherent cells were quantitated by cellular acid phosphatase assay (8). This assay measured acid phosphatase in ECV 304 cells although it was reported not detectable by a less sensitive immunochemistry technique (14). Per cent of inhibition of cell adhesion was determined after subtracting a background value obtained in ELISA. The effect of anti-integrin $\alpha_{IIb}\beta_3$ and anti-integrin β_1 antibodies on adhesion of HEL, ECV 304, and HF cell lines was tested by incubating cells with antibodies for 30 minutes at room temperature and then plating cells (10^5cell/well) and incubating for 4 hours at 37°C. After rinsing, adherent cells were quantified as above.

Detachment of adherent cells was analyzed by a modified procedure (20) using fibrinogen-coated microtiter plates, seeded with PMA-stimulated HEL cells or ECV 304 cells (10^5 cells/well). After incubation with tested peptides for 4 hours at 37°C, wells were rinsed and adherent cells were quantitated as described above.

Cell-Permeable Peptide Import Detection

Import of cell-permeable peptides was analyzed by confocal laser scanning microscopy of cells cytocentrifuged onto glass slides. Adherent cells were fixed with 3.5% paraformaldehyde, permeabilized with 0.25% Triton X-100, and reacted with respective monospecific antipeptide antisera for 1 hour at room temperature. Intracellular peptide-antibody complex was detected with rhodamine-conjugated anti-rabbit IgG (Kirkgaard & Perry). A Leitz confocal laser scanning microscope system was used with a 100X oil immersion lens as previously described (10). Alternatively, the import of peptides was quantitated by a Cell Enzyme-Linked Immunosorbent assay (Cell ELISA). Briefly, cells incubated with cell-permeable peptides (3-30μM) were washed, suspended in fresh medium and allowed to adhere to microtiter plates. After fixation and permeabilization, the cells were treated with monospecific antipeptide antibodies. Intracellular peptide-antibody complexes were detected with anti-rabbit IgG conjugated with alkaline phosphatase, and quantitated in ELISA.

Structure-Function Analysis of the Integrin β_3 Cytoplasmic Tail in HEL and ECV 304 Cells Using Cell-Permeable Peptides

For structure-function analysis of the cytoplasmic tail of integrin β_3, we synthesized four overlapping peptide analogs as specified in Figure 1. These peptides had no measurable effect on adhesion of PMA-stimulated HEL cells or ECV 304 cells to immobilized fibrinogen (Figure 2). However, when these peptides were rendered cell-permeable (10) through the addition of the hydrophobic (h) region sequence derived from the integrin β_3 signal peptide, they entered the cells and selectively exerted an inhibitory effect on cell adhesion to immobilized fibrinogen (Figure 2). Cell-permeable peptides were not cytotoxic within the concentrations used ($\leq$200μM), as determined by Trypan Blue exclusion.

The cell-permeable peptide β_3-1S, carrying the residues 747-762 of the β_3 cytoplasmic tail (SEQ ID NO:11), almost completely blocked the adhesion of both cell types to immobilized fibrinogen. In contrast, the cell-permeable peptides β_3-2S, β_3-3S, and β_3-4S were without measurable effect on cell adhesion. This structure-function analysis with cell-permeable peptides from the integrin β_3 cytoplasmic tail indicates that carboxy-terminal residues 747-762 (SEQ ID NO:11) constitute a functionally important sequence of the integrin β_3 cytoplasmic tail in two different cell types representing megakaryocytic and endothelial lineages. None of the tested peptides induced detachment of PMA-stimulated HEL cells when added 30 minutes after they were adherent to immobilized fibrinogen. Likewise, the tested peptides did not induce detachment of established monolayers of ECV 304 cells. All cell-permeable peptides were equally imported to the cytoplasm of HEL cells, as verified by confocal laser scanning microscopy following immunofluorescent staining with a peptide-specific antibody (Figure 3) and by quantitative analysis of imported peptides in cell ELISA of HEL and ECV 304 cells.

Inhibition of Cell Adhesion by Cell-Permeable Peptides is Integrin-Specific and Concentration-Dependent

Using integrin-specific antibodies, we determined that adhesion of HEL and ECV 304 cells to immobilized fibrinogen was mediated by integrin β_3 heterodimers

because anti-human integrin $\alpha_{IIb}\beta_3$ polyclonal antibody completely inhibited cell adhesion, while anti-human integrin β_1 antibody was without effect. On the other hand, adhesion of human fibroblasts to plastic was mediated by integrin β_1 heterodimers because anti-integrin β_1 inhibited adhesion, whereas anti-integrin $\alpha_{IIb}\beta_3$ antibody was without effect. Consistent with these results, cell-permeable peptide β_3-1S representing residues 747-762 of the cytoplasmic domain of integrin β_3 (SEQ ID NO:11) inhibited adhesion of HEL and ECV 304 cells to immobilized fibrinogen. (Fig. 4A and 4B). The cell-permeable β_1-1S peptide representing residue 788-803 of the cytoplasmic domain of integrin β_1 (SEQ ID NO:12) (15, 16) was non-inhibitory toward adhesion of HEL and ECV 304 cells to immobilized fibrinogen (Fig. 4A and Fig. 4B). On the other hand, adhesion of human fibroblasts to plastic mediated by integrin β_1 heterodimers was inhibited (75%) by cell-permeable β_1-1S peptide (200 μM), whereas cell-permeable β_3-1S peptide was inactive (Fig. 4C). These peptides were equally imported to HF cells as verified by cell ELISA (results not shown). In addition to the integrin-specific effects, the dose-response analysis indicates that the extracellular β_3-1S peptide concentrations required for 50% inhibition (EC_{50}) were 60μM and 55μM for HEL and ECV 304 cells, respectively. The EC_{50} of the cell permeable β_1-1S peptide in HF was 115μM. Because approximately 4% of cell-permeable peptide added to cells can be detected intracellularly (10) we estimate that intracellular peptide concentration causing 50% inhibition varies between 1-4μM. The cytoplasmic domains of human integrin β_1 and β_3 appear to be structurally similar (15, 16) as seven out of 16 residues in integrin β_1 segment (788-803) are identical with a corresponding sequence 747-762 of integrin β_3 (Figure 1). Since integrin β_3-mediated cell adhesion is inhibited from within by integrin β_3 peptide and integrin β_1- mediated adhesion is inhibited by integrin β_1 peptide, this pattern of inhibition indicates that regulation of the adhesive function of the integrin β_3 heterodimers in HEL and ECV 304 cells and integrin β_1 heterodimers in human fibroblasts follows an integrin-specific mechanism.

Cell-Permeable Mutant Peptides Identify Key Residues Involved in Regulation of Cell Adhesion

A "loss of function" point mutation $Ser^{752}Pro$ in the cytoplasmic segment of integrin β_3 is responsible for a life-long bleeding tendency and the abnormal adhesive function of integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) expressed in platelets of a Glanzmann thrombasthenia patient (5). This mutation lies in the functionally important segment of the integrin β_3 cytoplasmic tail identified in our experiments. Therefore, the question arises whether a $Ser^{752}Pro$ substitution in the functionally active cell-permeable peptide β_3-1S will result in a loss of its inhibitory function. Indeed, when the β_3-1S peptide had a $Ser^{752} \rightarrow Pro^{752}$ substitution, it lost inhibitory potency in a HEL and ECV 304 cell adhesion assay (Figure 5). To discern whether the $Ser^{752}Pro$ mutation is responsible for loss of function due to the lack of a potential phosphorylation site or due to the possible disruption by proline of the secondary structure of the β_3 integrin cytoplasmic tail, a second cell-permeable peptide with a $Ser^{752}Ala$ mutation (SEQ ID NO:15) was tested. This mutant peptide inhibited HEL and ECV 304 cell adhesion similarly to its wild-type β_3-1S analog (Figure 5). Thus, a proline-imposed effect on the secondary structure of the integrin β_3 cytoplasmic tail, rather than the loss of a potential phosphorylation site, can account for the observed differences. On the other hand, two tyrosine mutations in β_3-1S peptide involving conservative replacements $Tyr^{747}Phe$ and/or $Tyr^{759}Phe$ resulted in the loss of inhibitory function of β_3-1S peptide (Fig.6). Tyrosines 747 and 759 are therefore critically important for the inhibitory activity of the cell-permeable β_3-1S peptide. They constitute a functionally active tandem required for regulating the adhesive function of integrin β_3 in two different cell types. The role of phosphorylation of Tyr^{747} and Tyr^{759} in the function of β_3-1S peptide remains to be determined.

The results presented here indicate that the sequence 747-762 in integrin β_3 cytoplasmic tail (SEQ ID NO:2) constitutes the Cell Adhesion Regulatory Domain (CARD). The CARD of β_1 is found in amino acids 788-803 (SEQ ID NO:6). The sequence 724-769 in integrin β_2 cytoplasmic tail (SEQ ID Nos:9, 8, 7) contains an inhibitory peptide of β_2. The sequence 989-1008 in integrin α_{IIb} cytoplasmic tail (SEQ ID NO:10) represents the CARD of α_{IIb}. Although other motifs such as the conserved membrane-proximal short sequences present in the cytoplasmic "hinge" of integrins α_{IIb} and β_3 may also be involved (21), our structure-function analysis with a panel of cell-

permeable peptides suggests that CARD plays a pivotal role in the cell adhesive function of integrin β_3. Moreover, a homologous segment in integrin β_1 appears to regulate adhesion of human fibroblasts mediated by integrin β_1 heterodimers. Thus CARD is involved in integrin-specific regulation of cell adhesion. Our results transcend previous experiments with transiently expressed $\alpha_{IIb}\beta_3$ in heterologous CHO cells (9). In that study, the entire β_3 cytoplasmic tail was truncated, thereby abolishing cell spreading and adhesion mediated by $\alpha_{IIb}\beta_3$ recruited to focal adhesions. Regulation of binding of monoclonal antibody PAC1 to CHO cells doubly transfected with integrin $\alpha_{IIb}\alpha_{6B}$ and β_3 or β_1 chimeras appears to involve the NPXY motif (22). This motif is found in many integrin β subunits and is implicated in integrin localization in focal adhesions (23), in cleavage of the integrin β_3 cytoplasmic tail by calpain (24), in internalization of other membrane receptors (25); and in binding of a novel phosphotyrosine-binding (PTB) domain (26). However, two distinct cell-permeable peptides β_3-2S and β_3-4S that contained the ^{744}NPLY^{747} motif did not inhibit adhesion of HEL and ECV304 cells in our experimental system. This finding together with results of Tyr^{747}Phe and Tyr^{759}Phe mutations within the β_3-1S peptide support the proposal that the two tyrosines (747 and 759), acting in tandem within CARD, are essential for regulation of the adhesive function of integrin β_3. Similarly spaced tyrosines play a role in the interaction of T and B cell antigen receptor cytoplasmic tails (27). The functionally active β_3-1S peptide imported to HEL and ECV 304 cells can exert its inhibitory effect by interacting with α_{IIb} or α_v integrins, respectively. Alternatively, the β_3-1S peptide can interact with other CARD-recognizing cytoplasmic proteins, e.g. β_3 endonexin (28). The identity of cytoplasmic protein(s) interacting with CARD remains to be established.

Cell-Permeable Peptide Turnover Is Regulated by the Multidrug Resistance Pump (P-Glycoprotein).

The membrane P-glycoprotein (P170; mdr1 gene product) is an ATP-hydrolyzing transmembrane pump. It is responsible for multidrug resistant phenotype by its capacity to prevent intracellular accumulation of unrelated chemotherapeutic drugs, usually of hydrophobic nature (57). It is possible to reverse the function of the multidrug resistance pump by three categories of compounds: chemotherapeutic drug

analogs, calcium channel antagonists (*e.g.*, verapamil), immunosuppressive cyclic peptides (*e.g.*, cyclosporins) and calmodulin inhibitors **(57)**. They reverse MDR by inhibiting drug efflux and therefore increasing accumulation of drug in MDR cells. Similarly, drug accumulation is observed in mdr gene knockout mice **(58)**. To test our hypothesis that MDR pump is involved in regulation of intracellular level of cell-permeable peptides imported to HEL cells we used MDR pump-reversing agent, verapamil. Cell-permeable peptide β_3-1S known to inhibit adhesion of phorbol ester-stimulated HEL cells to immobilized fibrinogen was assayed in the absence and presence of verapamil. The extracellular concentration of β_3-1S peptide necessary to cause 50% inhibition of adhesion (EC_{50}) in HEL cells in 80μM. When cells were pretreated with verapamil (1 μM) the EC_{50} of β_3-1S peptide was reduced to 15μM. This means that effective intracellular concentration of cell-permeable β_3-1S peptide was 6 times higher when MDR pump was reversed by verapamil (Fig. 8).

HEL cells treated with verapamil alone had unimpaired adhesion and there is no indication the verapamil inhibited cell-permeable peptide import to cells. These experiments indicate that the MDR pump is involved in regulation of cell-permeable peptide efflux. When the efflux is blocked, cell-permeable peptide accumulates in HEL cells thereby exerting more pronounced inhibitory effect on intracellular protein-protein interactions involved in regulation of adhesive function of integrins β_3. Thus, cell-permeable peptides can move in and out of the cells. These results are consistent with reports from other laboratories that MDR pump is involved in binding a number of intracellular peptides **(59), (60).**

In summary, structure-function analysis of the intracellular segment of integrins using cell-permeable peptides pinpoints the Cell Adhesion Regulatory Domains. Inhibition of integrin $\alpha_{IIb}\beta_3$-mediated cell adhesion to immobilized fibrinogen by functionally active cell-permeable peptides is an alternative to pharmacologic blockade of the extracellular, ligand-binding domains of this integrin (29). Imported β_3 peptides compete with the endogenous integrin β_3 cytoplasmic tail to interrupt integrin-specific intracellular protein-protein interactions that engage the cytoplasmic "business end" of

integrin β_3. Moreover, CARD representing integrin β_1 cytoplasmic segment can inhibit adhesion of human fibroblasts in integrin specific manner. This approach offers a unique opportunity to modulate the adhesive functions of cellular integrins "from within." Use of the import of a CARD peptide into cells is enhanced by also administering a composition comprising a compound that can block the export of peptides from a particular cell, such as a calcium channel blocker.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

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SEQUENCE LISTING

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(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Val Thr Val Leu Ala Leu Gly Ala Leu Ala Gly Val Gly Val Gly
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Tyr Lys Glu Ala Thr Ser Thr Phe Thr Asn Ile Thr Tyr Arg Gly Thr
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ala Arg Ala Lys Trp Asp Thr Ala Asn Asn Pro Lys Tyr Lys Glu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

His Asp Arg Lys Glu Phe Ala Lys Phe Glu Glu Glu Arg Ala Arg Ala
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ala Asn Asn Pro Leu Tyr Lys Glu Ala Thr Ser Thr Phe Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Tyr Lys Ser Ala Val Thr Thr Val Val Asn Pro Lys Tyr Glu Gly Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Lys Ser Ala Thr Thr Thr Val Met Asn Pro Lys Phe Ala Glu Ser
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Lys Glu Lys Leu Lys Ser Gln Trp Asn Asn Asp Asn Pro Leu Phe Lys
1 5 10 15
Ser

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Lys Ala Leu Ile His Leu Ser Asp Leu Arg Glu Tyr Arg Arg Phe Glu
1 5 10 15
Lys Glu

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Lys Val Gly Phe Phe Lys Arg Asn Arg Pro Pro Leu Glu Glu Asp Asp
1 5 10 15
Glu Glu Gly Glu
20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Val Thr Val Leu Ala Leu Gly Ala Leu Ala Gly Val Gly Val Gly Tyr
 1               5                  10                  15
Lys Glu Ala Thr Ser Thr Phe Thr Asn Ile Thr Tyr Arg Gly Thr
            20                  25                  30
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Val Thr Val Leu Ala Leu Gly Ala Leu Ala Gly Val Gly Val Gly Tyr
 1               5                  10                  15
Lys Ser Ala Val Thr Thr Val Val Asn Pro Lys Tyr Glu Gly Lys
            20                  25                  30
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Val Thr Val Leu Ala Leu Gly Ala Leu Ala Gly Val Gly Val Gly Lys
 1               5                  10                  15
```

Ser Ala Thr Thr Thr Val Met Asn Pro Lys Phe Ala Glu Ser
20 25 30

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Val Thr Val Leu Ala Leu Gly Ala Leu Ala Gly Val Gly Val Gly Lys
1 5 10 15
Val Gly Phe Phe Lys Arg Asn Arg Pro Pro Leu Glu Glu Asp Asp Glu
20 25 30
Glu Gly Glu
35

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Tyr Lys Glu Ala Thr Ala Thr Phe Thr Asn Ile Thr Tyr Arg Gly Thr
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Val Thr Val Leu Ala Leu Gly Ala Leu Ala Gly Val Gly Val Gly Tyr
1 5 10 15
Lys Glu Ala Thr Ala Thr Phe Thr Asn Ile Thr Tyr Arg Gly Thr
20 25 30

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Lys Ala Leu Ile His Leu Ser Asp Leu Arg Glu Tyr Arg Arg Phe Glu
1 5 10 15
Lys Glu Lys Leu Lys Ser Gln Trp Asn Asn Asp Asn Pro Leu Phe Lys

```
                20                  25                  30
Ser Ala Thr Thr Thr Val Met Asn Pro Lys Phe Ala Glu Ser
        35                  40                  45
```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not applicable
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
His Asp Arg Lys Glu Phe Ala Lys Phe Glu Glu Glu Arg Ala Arg Ala
 1               5                  10                  15
Lys Trp Asp Thr Ala Asn Asn Pro Lys Tyr Lys Glu Ala Thr Ser Thr
            20                  25                  30
Phe Thr Asn Ile Thr Tyr Arg Gly Thr
        35                  40
```

SEQ ID NO: 20: peptide

LFKSATTTVMNPKFAES

SEQ ID NO: 21: peptide

KEKLKSQWNNDNPLF

What is claimed is:

1. A method of inhibiting adhesion of a cell comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by the cell.

2. The method of claim 1, wherein the adhesion receptor is an integrin.

3. The method of claim 2, wherein the subunit is a β subunit of an integrin.

4. The method of claim 3, wherein the β subunit is β_3.

5. The method of claim 3, wherein the β subunit is β_2.

6. The method of claim 3, wherein the β subunit is β_1.

7. The method of claim 2, wherein the subunit is an α subunit of an integrin.

8. The method of claim 7, wherein the α subunit is α_{IIb}.

9. The method of claim 7, wherein the α subunit is α_L.

10. The method of claim 7, wherein the α subunit is α_M.

11. The method of claim 7, wherein the α subunit is α_X.

12. The method of claim 1, wherein the adhesion receptor is a selectin.

13. The method of claim 12, wherein the selectin is L-selectin.

14. The method of claim 12, wherein the selectin is E-selectin.

15. The method of claim 12, wherein the selectin is P-selectin.

16. The method of claim 1, wherein the adhesion receptor is a cadhesin.

17. The method of claim 16, wherein the cadhesin is E-cadhesin.

18. The method of claim 16, wherein the cadhesin is N-cadhesin.

19. The method of claim 16, wherein the cadhesin is P-cadhesin.

20. The method of claim 1, wherein the counter receptor is an ICAM.

21. The method of claim 20, wherein the ICAM is ICAM-1.

22. The method of claim 20, wherein the ICAM is ICAM-2.

23. The method of claim 20, wherein the ICAM is ICAM-3.

24. The method of claim 1, wherein the cell is a cancer cell.

25. The method of claim 1, wherein the cell is a fibroblast and the adhesion receptor is an integrin comprising subunit β_1.

26. The method of claim 1, wherein the peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor is transferred into the cell by contacting the cell with a chimeric peptide comprising an importation-competent signal peptide at the N-terminus of the chimeric peptide and the cell adhesion regulatory domain, thereby transferring the peptide and inhibiting adhesion of the cell.

27. The method of claim 26, wherein the cell is contacted with the chimeric peptide and a compound that blocks a cellular membrane channel exporting peptides and drugs to outside the cell.

28. The method of claim 27, wherein the compound is verapamil.

29. The method of claim 27, wherein the compound is a cyclosporin.

30. The method of claim 1, wherein the peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor is transferred into the cell by contacting the cell with a complex comprising the cell adhesion regulatory domain linked to an importation-competent signal peptide, thereby transferring the peptide and inhibiting adhesion of the cell.

31. A method of reducing or preventing an inflammatory response of a cell comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by the cell.

32. The method of claim 31, wherein the adhesion receptor is an integrin.

33. The method of claim 32, wherein the subunit is a β subunit of an integrin expressed by the cell.

34. The method of claim 32, wherein the subunit is an α subunit of an integrin expressed by the cell.

35. A method of reducing or preventing excessive proliferation of a fibroblast comprising transferring into the fibroblast a peptide comprising a cell adhesion regulatory domain of a β_1 subunit of an integrin receptor.

36. The method of claim 35, wherein the peptide comprises the amino acid sequence set forth in SEQ ID NO:6.

37. The method of claim 35, wherein the peptide comprises the amino acid sequence set forth in SEQ ID NO:12.

38. The method of claim 35, wherein the reduction or prevention is sufficient to effect wound healing.

39. A method of reducing or preventing blood clotting in a subject comprising contacting a platelet with a chimeric peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, thereby reducing or preventing platelet cell adhesion and reducing or preventing blood clotting in the subject.

40. The method of claim 39, wherein the β subunit is β_3.

41. The method of claim 39, wherein the peptide comprises the amino acid sequence set forth in SEQ ID NO:2.

42. A method of reducing or preventing platelet adhesion in extracorporeal circulation of a subject comprising contacting *ex vivo* during the extracorporeal circulation process a platelet from the subject with a chimeric peptide comprising a signal peptide at the N-terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, and replacing the platelet into the subject, thereby reducing or preventing platelet cell adhesion in the extracorporeal circulation.

43. A method of reducing or preventing platelet cell adhesion in a subject comprising contacting a platelet with a chimeric peptide comprising a signal peptide at the N-

terminus of the chimeric peptide and a cell adhesion regulatory domain of a β subunit of an integrin, thereby reducing or preventing platelet cell adhesion in the subject.

44. A method of treating or preventing coronary and/ or vascular disease or conditions in a subject comprising administering to the subject a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by a vascular endothelial cell, thereby reducing or inhibiting attachment of leukocytes and platelets to an atherosclerotic lesion.

45. A method of reducing or preventing granulocyte adhesion in a subject comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by the granulocyte, thereby reducing or preventing adhesion of the granulocyte in the subject.

46. A method of preventing or reducing restenosis in the blood vessels of a subject, comprising administering to the subject comprising transferring into the cell a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by an endothelial cell of the blood vessel, a granulocyte or a platelet, thereby reducing or preventing adhesion of the endothelial cells, granulocyte, and/or platelet in the subject.

47. A method of treating adult respiratory distress syndrome in a subject comprising administering to the subject a peptide comprising a cell adhesion regulatory domain of a subunit of an adhesion receptor or counter receptor expressed by a granulocyte of the subject, thereby preventing or reducing adhesion of a granulocyte to the endothelial lining of the microvasculature of the lungs and treating adult respiratory distress syndrome.

48. A peptide comprising a cell adhesion regulatory domain of a β subunit of an integrin.

49. The peptide of claim 48, wherein the β subunit is β_1.

50. The peptide of claim 49, wherein the β subunit comprises amino acids 788-803 of β_1 subunit.

51. The peptide of claim 48, wherein the β subunit is β_3.

52. The peptide of claim 51, wherein the β subunit comprises amino acids 747-762 of β_3 subunit.

53. The peptide of claim 48, wherein the β subunit is β_2.

54. The peptide of claim 53, wherein the β subunit comprises amino acids 724-769 of β_2 subunit.

55. The peptide of claim 53, wherein the β subunit has a Ser^{752} to Ala^{752} substitution.

56. A peptide comprising a cell adhesion regulatory domain of an α subunit of an integrin.

57. A peptide comprising a signal peptide linked at the N-terminus to a cell adhesion regulatory domain of a β subunit of an integrin.

58. A peptide comprising a signal peptide linked at the N-terminus to a cell adhesion regulatory domain of an α subunit of an integrin.

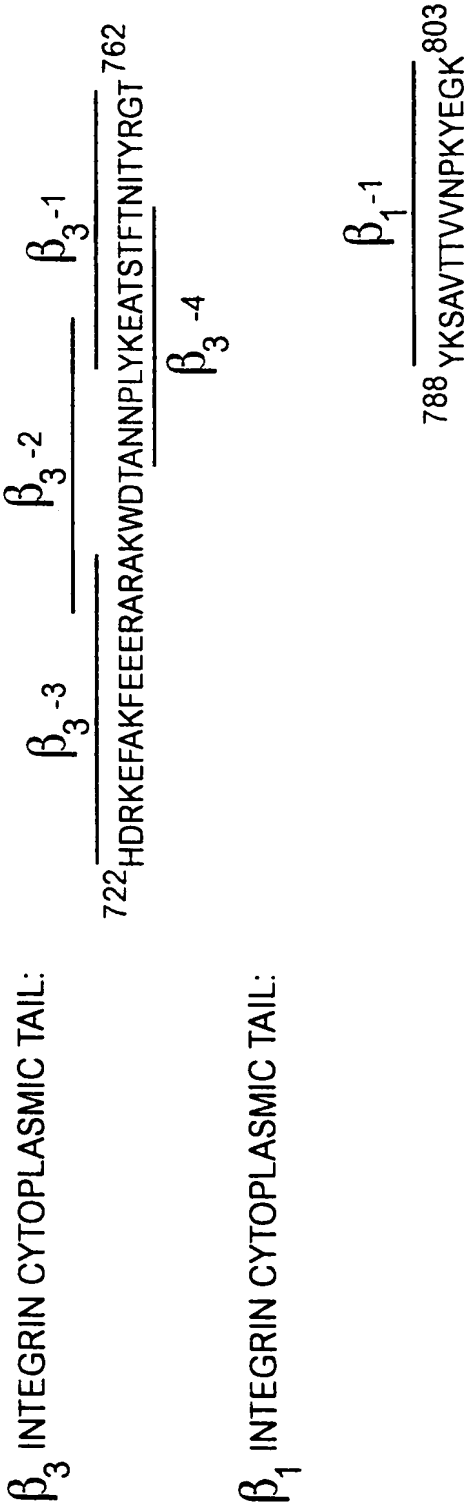


FIG.1

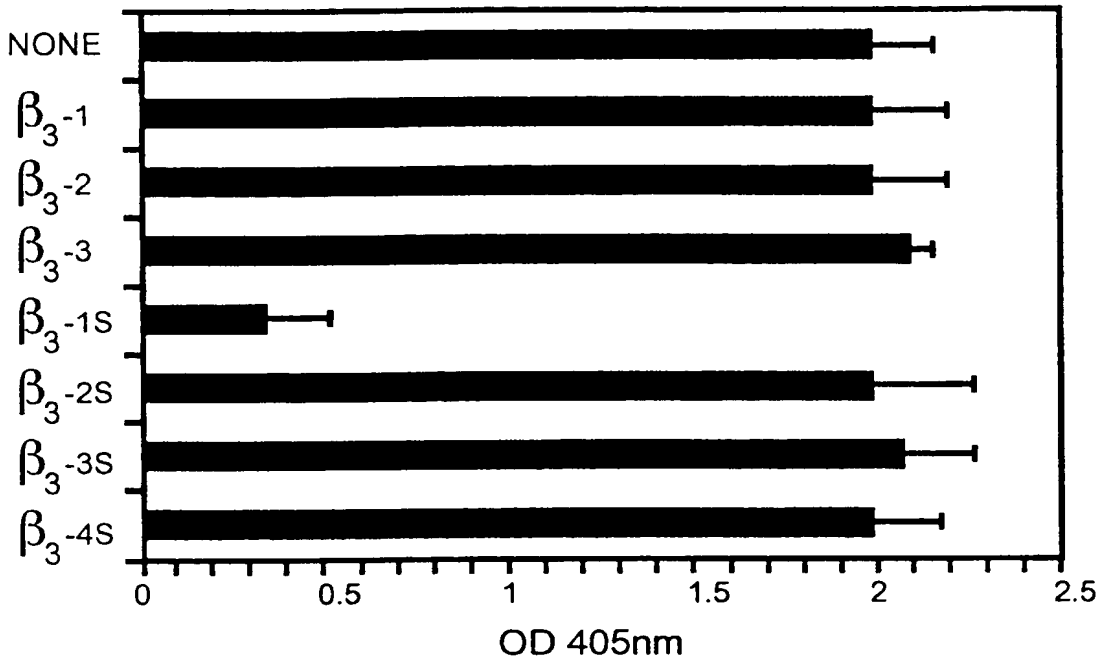


FIG.2A

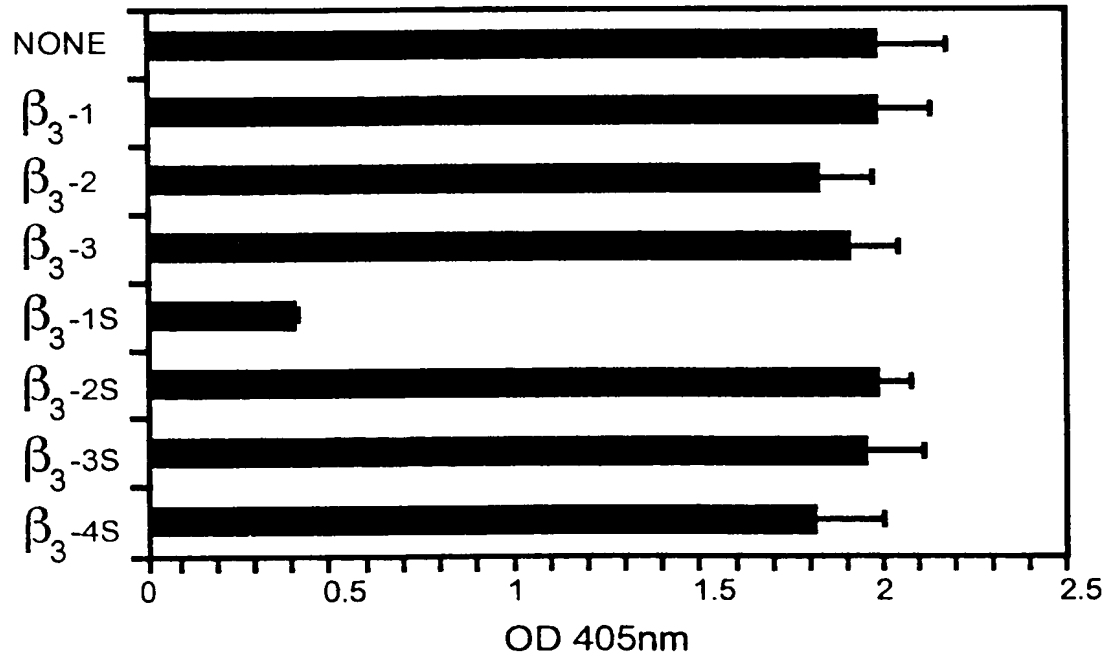
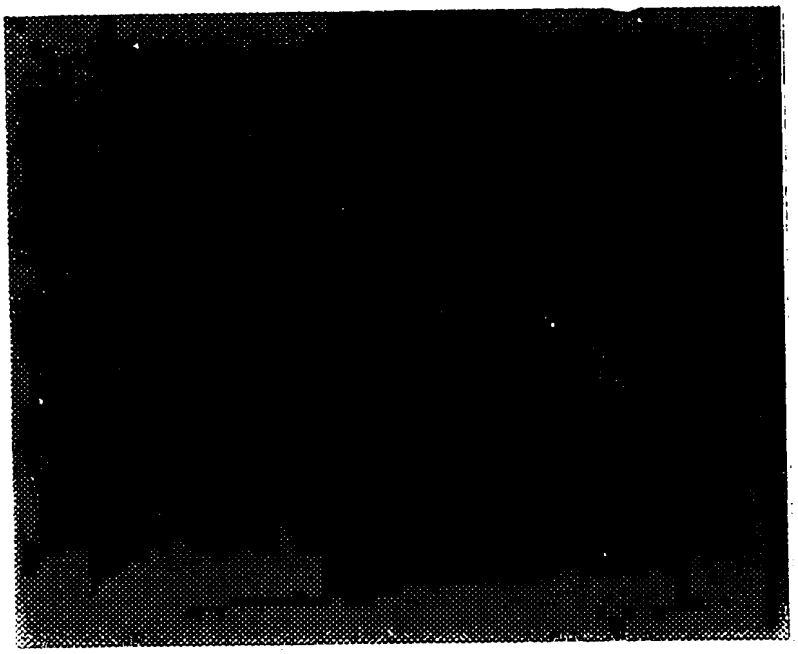


FIG.2B



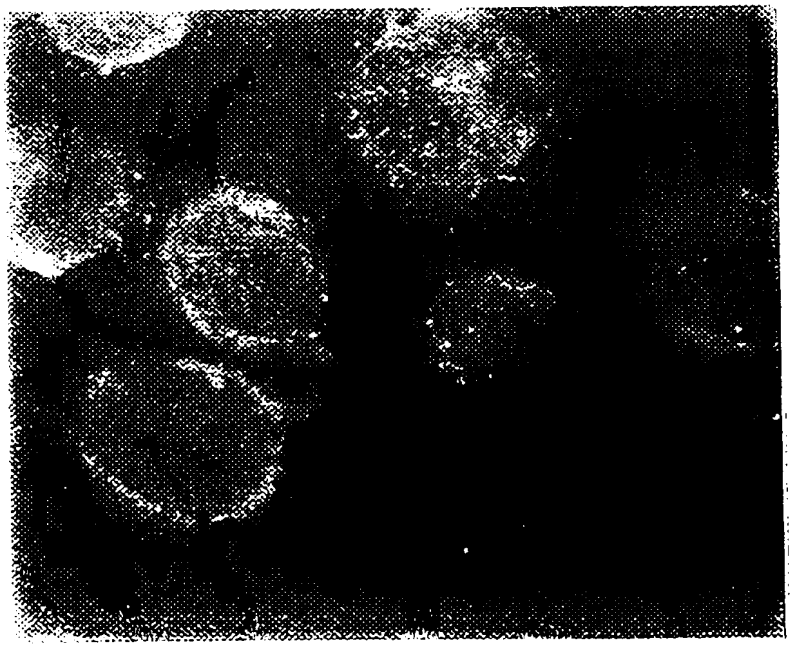


FIG 3

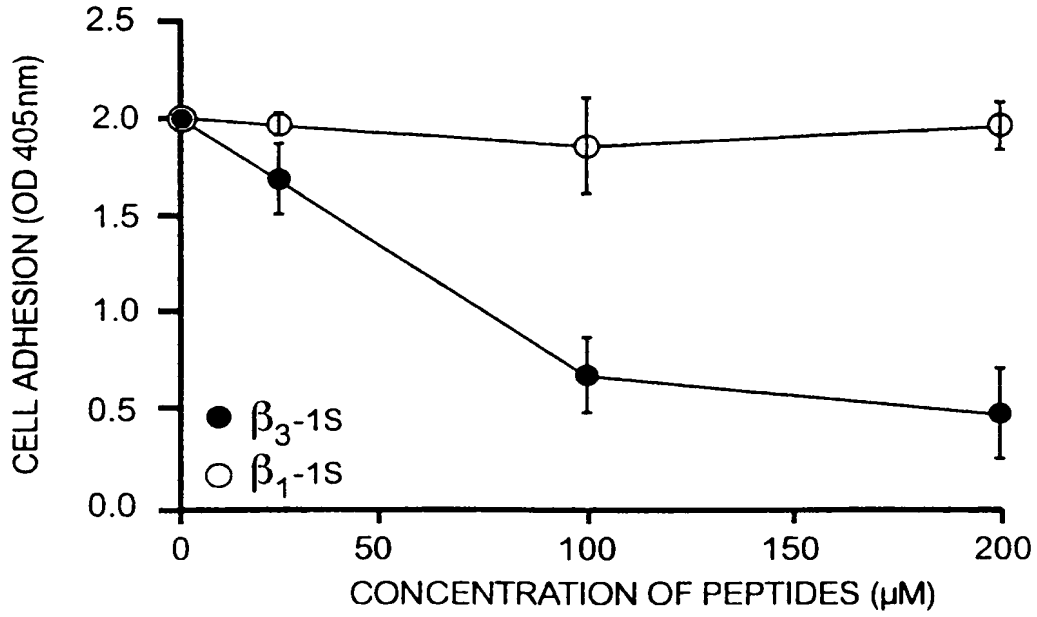


FIG.4A

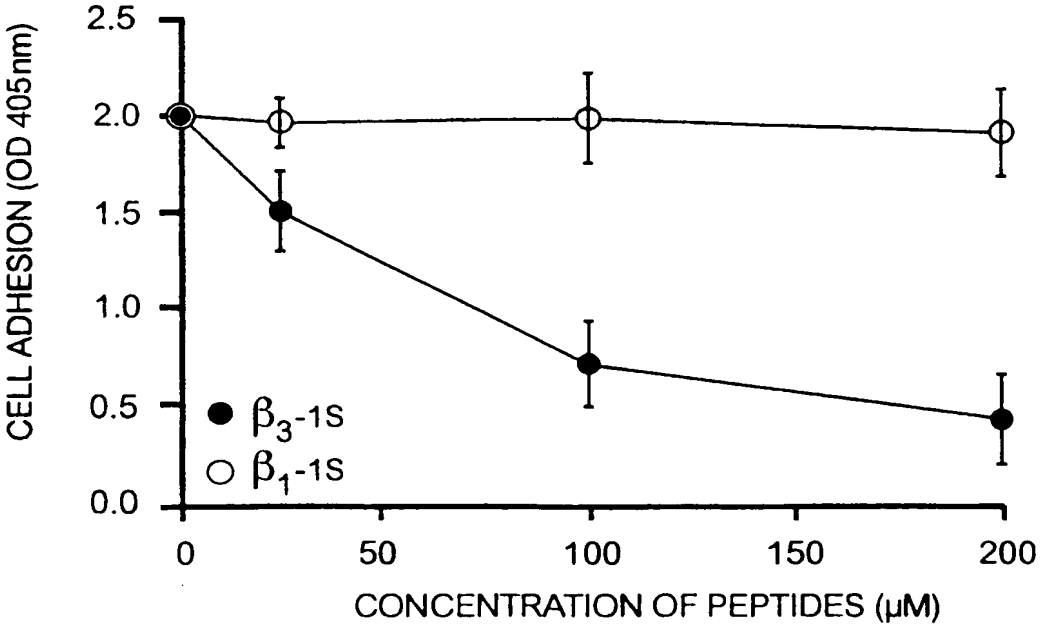


FIG.4B

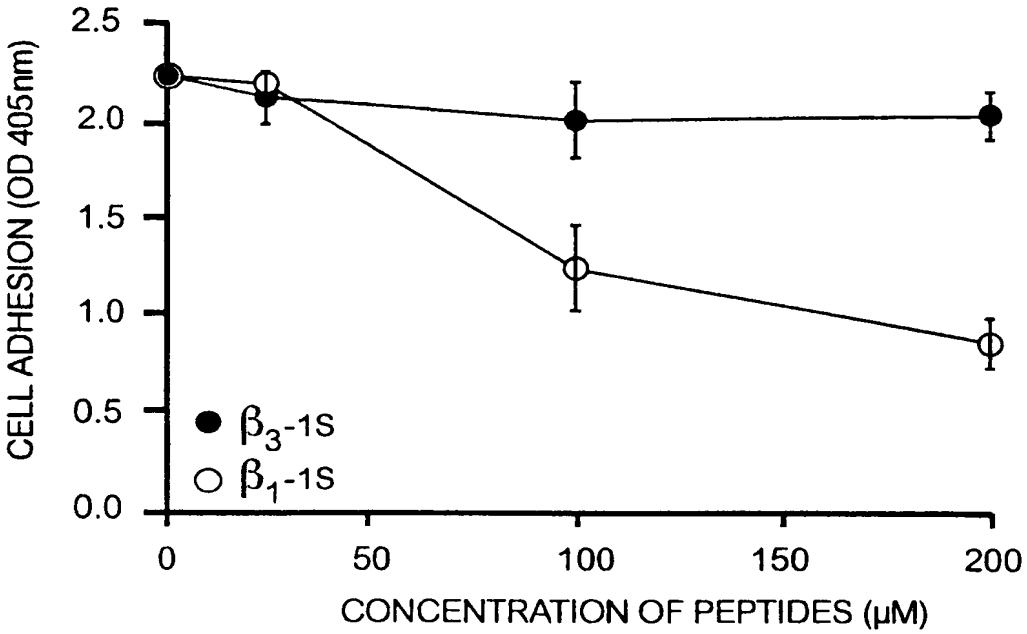


FIG.4C

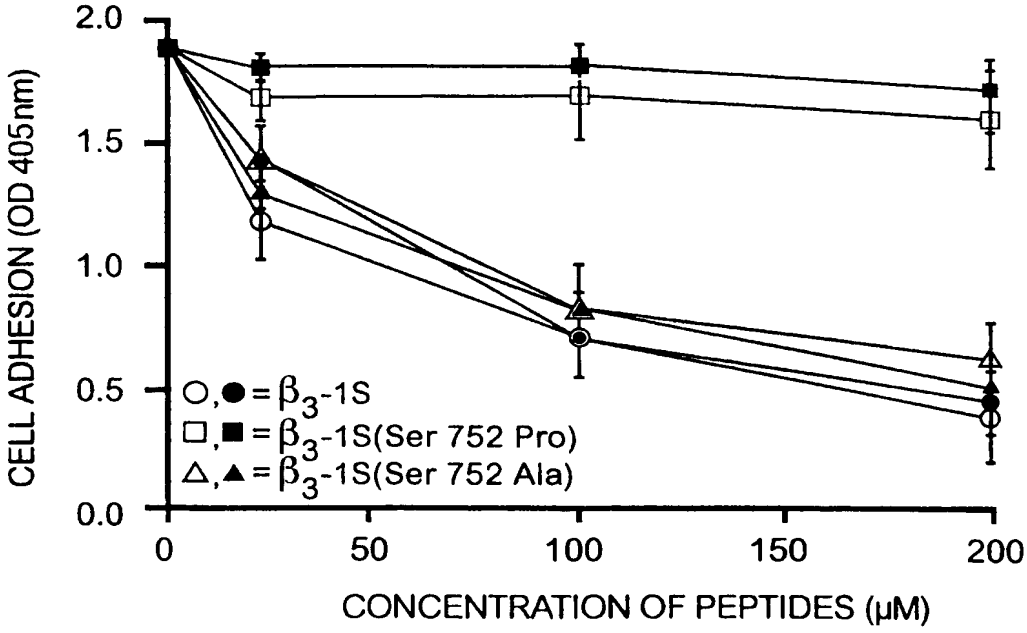


FIG.5

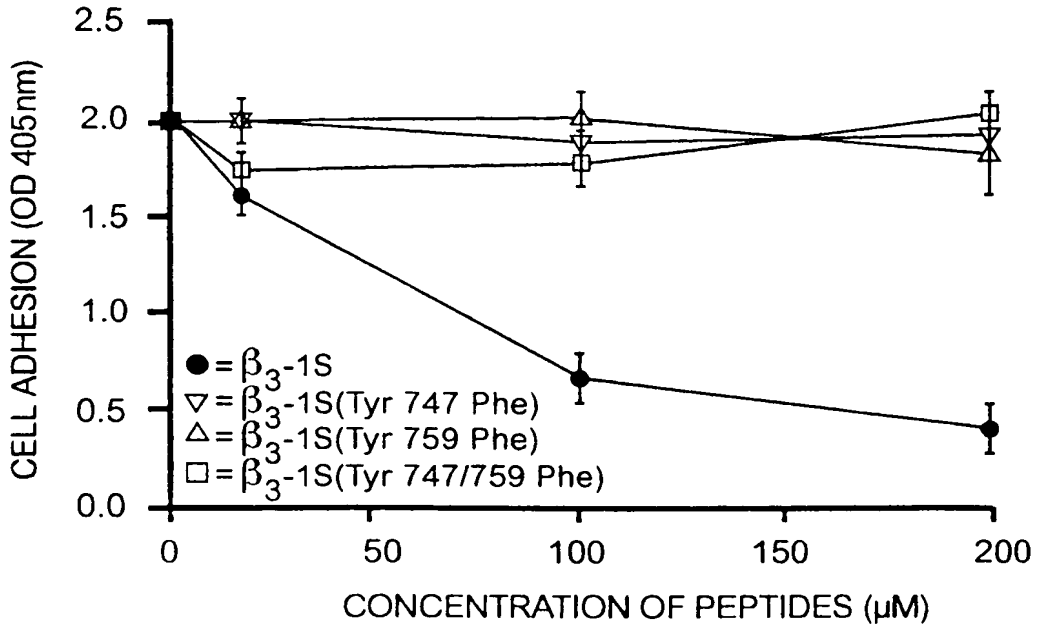


FIG.6A

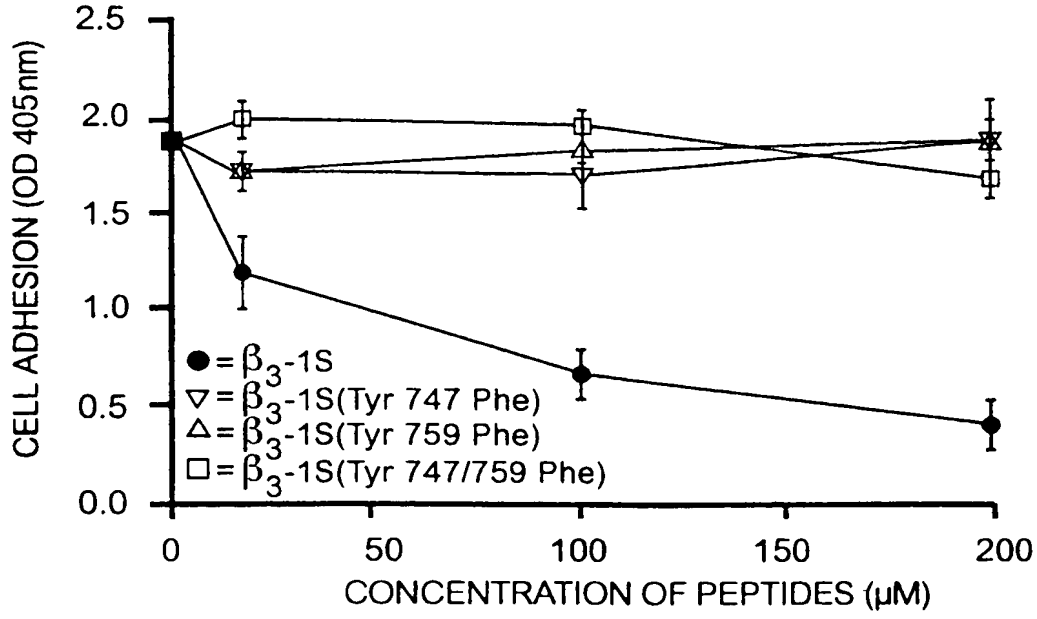


FIG.6B

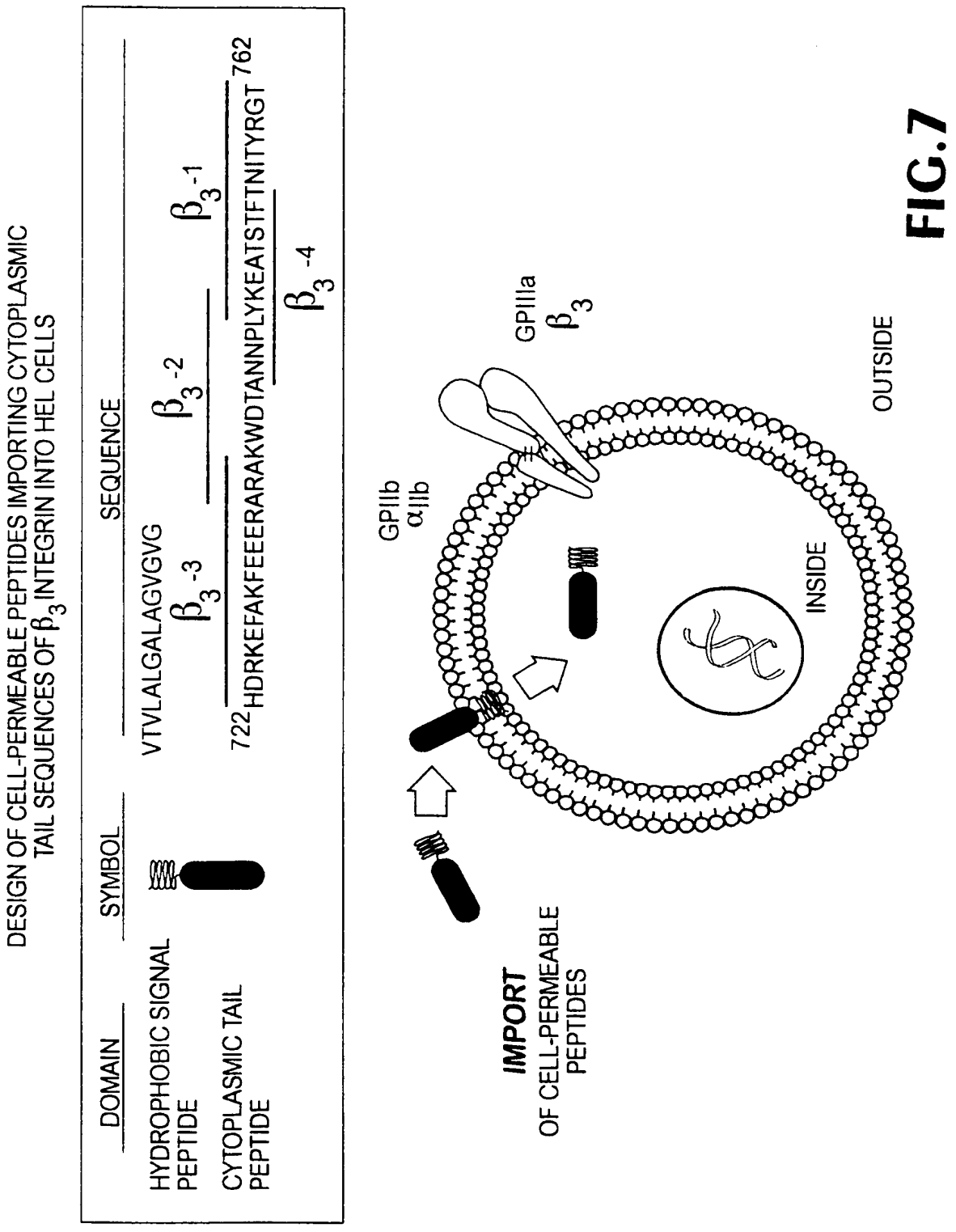


FIG.7

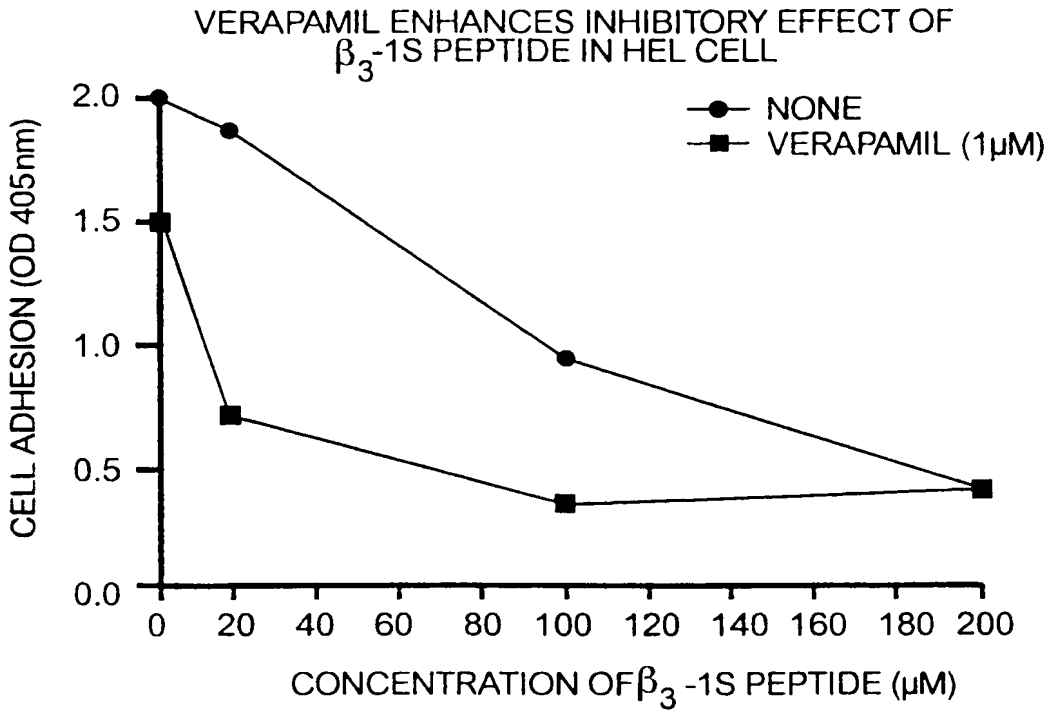


FIG.8

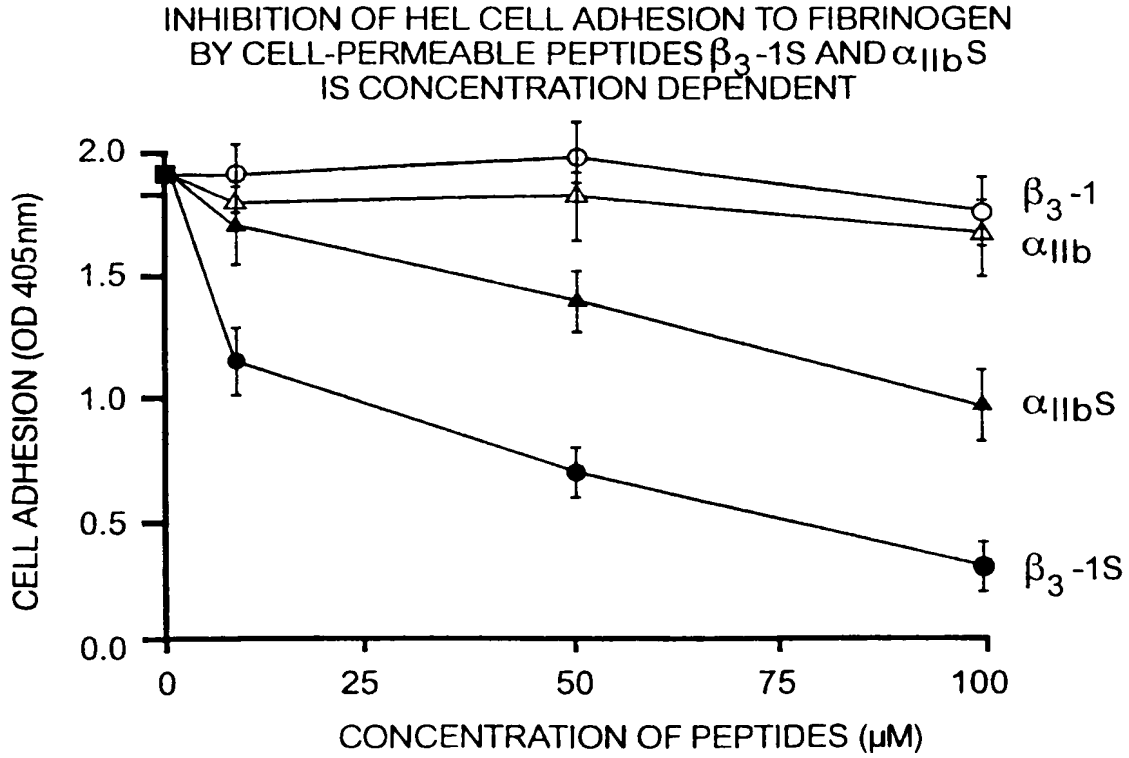


FIG.9

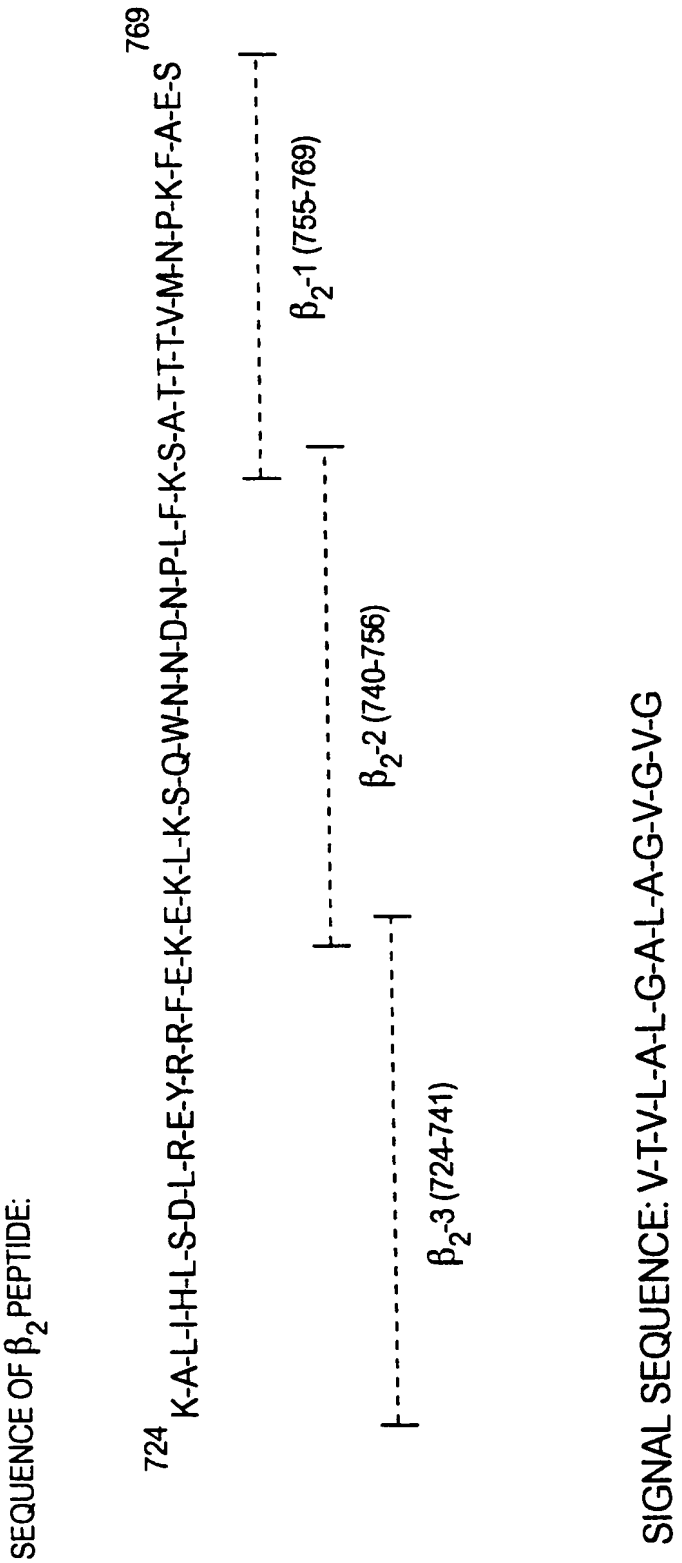


FIG.10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18331

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/10, 38/16, 31/335, 31/135; C07K 7/08, 14/47

US CL :514/2, 12, 450, 646; 530/300, 324, 326

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 12, 450, 646; 530/300, 324, 326

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS/USPAT, STN/Medline, CaPlus

search terms: beta, integrin#, peptide#, cytoplasm?, chimer?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LAFLAMME, S.E. et al. Single Subunit Chimeric Integrins as Mimics and Inhibitors of Endogenous Integrin Functions in Receptor Localization, Cell Spreading and Migration, and Matrix Assembly. September 1994. Journal of Cell Biology, Volume 126, Number 5, pages 1287-1298, especially pages 1292-1295.	1-4, 6, 25, 35-38, 48-52, 57
X ----- Y	LUKASHEV, M.E. et al. Disruption of Integrin Function and Induction of Tyrosine Phosphorylation by the Autonomously Expressed Beta 1 Integrin Cytoplasmic Domain. 15 July 1994. Journal of Biological Chemistry. Volume 269, Number 28, pages 18311-18314, especially Fig. 3 and pages 18312-18314.	1-3, 6, 25, 35-38, 48-50 ----- 5, 31-33, 45-46, 53-54

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

19 DECEMBER 1997

Date of mailing of the international search report

07 JAN 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18331

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,340,800 A (LIU et al) 23 August 1994, column 1, line 36 to column 3, line 15.	5, 31-33, 45-46, 53-54
P, X ------ P, Y	LIU, X.-Y. et al. Identification of a Functionally Important Sequence in the Cytoplasmic Tail of Integrin Beta 3 by Using Cell-Permeable Peptide Analogs. 15 October 1996. Proceedings of the National Academy of Science USA, Volume 93, pages 11819-11824, see entire document.	1-4, 6, 24-26, 31-33, 35-38, 48-52, 57 ----- 39-44, 46
Y	US 5,114,842 A (PLOW et al.) 19 May 1992, column 1, line 65 to column 2, line 52; and column 21, line 12 to column 22, line 42.	39-44, 46
A	LIN, Y.-Z. et al. Inhibition of Nuclear Translocation of Transcription Factor NF-Kappa B by a Synthetic Peptide Containing a Cell Membrane-Permeable Motif and Nuclear Localization Sequence. 16 June 1995. Journal of Biological Chemistry, Volume 270, Number 24, pages 14255-14258, see entire document.	1-6, 24-33, 35-55, 57
A	RUOSLAHTI, E. et al. Anchorage Dependence, Integrins and Apoptosis. 20 May 1994. Cell, Volume 77, pages 477-478, see entire document.	1-6, 24, 30, 35-38

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18331

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-6, 24-33, 35-55, 57

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18331

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:

Beta integrin subunits
Alpha integrin subunits
Selectins
Cadherins
ICAMs

The claims are deemed to correspond to the species listed above in the following manner:

Beta integrin subunits: claims 3-6, 25, 33, 35-43, 48-55, 57
Alpha integrin subunits: claims 7-11, 34, 56, 58
Selectins: claims 12-15
Cadherins: claims 16-19
ICAMs: claims 20-23

The following claims are generic: 1-2, 24, 26-32, 44-47

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons:

The invention recites a method of inhibiting cell adhesion by transferring a peptide comprising a cell adhesion regulatory domain derived from an adhesion protein into a cell. The special technical feature of the first species recited is the cell regulatory domain derived from a beta integrin subunit. This special technical feature is not shared with the species of the alpha integrin subunits, the selectins, the cadherins or the ICAMs because the cell regulatory domains of each of these species are completely different from the beta integrin subunit and from one another. The cell regulatory domain of each species are structurally different from each other and from the cell regulatory domain of the beta integrin subunits because each cell regulatory domain contains completely different amino acid sequences. Furthermore, each of these cell regulatory domains are functionally different from each other because the cell regulatory domains of each species interact with a different set of intracellular proteins in the claimed method. Therefore, since none of the other species' cell regulatory domains are structurally or functionally similar to that of the beta integrin subunits, these species do not share the same special technical feature with that of the beta integrin subunit. In addition, since none of the species' cell regulatory domains are structurally or functionally similar to each other's, these species also do not share a special technical feature with each other.